Systems Pharmacology and Blood-Brain Barrier Functionality in Parkinson's Disease

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The only true wisdom is in knowing you know nothing

Socrates (470 B.C. - 399 B.C.)

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Section I :
General Introduction

Chapter 1

Scope and Outline of the Investigations

1. General objective

The objective of the research described in this thesis was to explore rotenone as a toxin for inducing Parkinson's disease in rats as a new rat model of this disease, and to use this rat model in pharmacokinetic(-pharmacodynamic) (PK-PD) studies on antiparkinson drugs with special reference to blood-brain barrier (BBB) functionality.

2. Scope and outline

Parkinson's disease is a progressive neurodegenerative disease, characterised by the loss of dopamine producing neurons in the striatum and substantia nigra pars compacta (SNc). Dopamine controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. Pathological conditions such as Parkinson's disease are linked to a dysregulation of dopaminergic transmission (Missale *et al*., 1998), resulting in clinical symptoms like bradykinesia, resting tremors, rigidity and postural instabilities (Thomas and Beal, 2007). Dopamine is formed from L-3,4-dihydroxyphenylalanine (L-DOPA) and the treatment of Parkinson's disease consists mainly of symptomatic treatment by replacing the lost dopamine with L-DOPA. L-DOPA is given in combination with a peripheral aromatic amino acid decarboxylase (AAADC) inhibitor, while at later stages other drugs (such as direct dopamine receptor agonists, monoamine-oxidase (MAO) inhibitors, catechol-O-methyltransferase (COMT) inhibitors) may be added. Unfortunately, treatment of Parkinson's disease is not without complications as typically the effect of symptomatic drug treatment wears off with progression of the disease and motor complications arise. At present it is poorly understood which mechanisms contribute to the loss of efficacy. Moreover there is an increasing interest in the development of treatments aimed at impairing disease progression. An important aspect in research on drug treatment of Parkinson's disease is its multifactorial nature. For example there may be changes in the brain distribution of drugs, the disposition of endogenous neurotransmitters, the expression and functionality of receptors etc. This underscores the importance of a system's approach towards the development of novel drug treatment paradigms. The investigations in this thesis focus on alterations on BBB transport in relation to Parkinson's disease progression.

Overview of the pathophysiology of Parkinson's disease and the current treatment modalities, emphasis on changes in the functionality of the BBB

In **Chapter 2**, a summary of Parkinson's disease characteristics, diagnosis and etiopathogenesis is given. In Parkinson's disease, the functional imbalance in dopaminergic transmission is thought to be responsible for the bradykinesia, which may be temporarily normalised by dopamine replacement therapy. In the treatment of Parkinson's disease, L-DOPA in combination with a peripheral AAADC inhibitor like Carbidopa or Benserazide, is the golden standard. In a later stage of the disease, L-DOPA is often administered in combination with a dopamine agonist, a COMT inhibitor, or a MAO-B inhibitor to overcome any motor complications associated with long-term L-DOPA treatment. However, the further the progression of Parkinson's disease, the more effective drug treatment fails and more complications occur.

In order to be able to answer the questions on the reasons behind this reduction in effectiveness, one should consider the mechanisms that may affect the target site distribution of the CNS drug and ultimately the drug response (e.g. plasma protein binding, BBB transport, within brain distribution, target interaction). To improve drug treatment (and drug development), detailed information on the interrelationship between disease, pharmacokinetics and drug response is needed as all individual mechanisms may vary among different physiological, pathological, and chronic drug treatment conditions, and therewith their relative importance in relation to the effect. **Chapter 2** presents an overview of the mechanisms involved in drug response and the factors which influence these mechanisms. Special emphasis is on the contribution of the BBB on the drug response, specifically under diseased conditions like Parkinson's disease. BBB transport of a particular CNS drug into and out of the brain is the sum of all actual BBB transport mechanisms applicable to that particular drug. Therefore, any changes in BBB transport mechanisms may affect actual BBB transport and therewith the effects of the drug. This may also apply for the drugs used in treatment of Parkinson's disease, like L-DOPA as well as the co-administered AAADC inhibitors. This may contribute to the motor complications that develop after long-term treatment with L-DOPA in the more advanced stages of the disease. This implies the need for research on the role of the BBB in the dose-response relationships of the Parkinson's disease related drugs along with the stage of the disease.

Overview of experimental animal models of Parkinson's disease

In **Chapter 3**, an overview of the most commonly used animal models in Parkinson's disease research together with their main disease characteristics is presented. Further information is provided on *in vivo* techniques to assess target site distribution as well as on behavioural tests for pharmacodynamic information.

As Parkinson's disease is composed of many components -each caused by the interplay of a number of genetic and nongenetic causes- a systems pharmacology approach to the improvement of present drug treatments and the development of novel drug treatments of Parkinson's disease is warranted. Animal-based models combined with integrated research approaches that address the individual mechanisms involved, including their time-dependencies, are needed to fully understand these aspects.

The currently applied animal models for Parkinson's disease are presented as toxin-induced and genetic animal models. The behavioural tests that can be used in animal models include drug-induced rotometry, the staircase- and the rotorod test, among others. Further emphasis has been put on *in vivo* techniques to assess the BBB functionality on drug transport into and out of the brain, and the distribution of drugs to the target site. To that end, intracerebral microdialysis is very useful as it is able to measure kinetics of exogenous compounds. Moreover, it also offers the possibility of monitoring endogenous compounds such as neurotransmitters (e.g. dopamine) and its metabolites and any changes in their kinetics as a consequence of disease and/or treatment.

Taken together, information obtained experimentally by applying animal models of Parkinson's disease in combination with intracerebral microdialysis and behavioural testing can be used for the development of advanced mechanism-based PK-PD models.

Experimental research on the 'rotenone model' of Parkinson's disease

Chapter 4 deals with experimental research on the 'rotenone model' of Parkinson's disease. The aim was to find a suitable and applicable animal model for Parkinson's disease resembling the characteristics of the disease, specifically displaying slow and selective lesioning of dopaminergic neurons over time in addition to the presence of Lewy Bodies (LB) in the remaining neurons and detectable motor deficits. To that end, the neurotoxin rotenone was used for inducing Parkinson's disease in rats, and a comparison was made between the systemic and the intracerebral route of administration.

Previously, the administration of low-dose intravenous or subcutaneous rotenone to rats has been shown to produce a slow, selective degeneration of nigrostriatal dopaminergic neurons accompanied by α-synuclein-positive LB inclusions as seen in Parkinson's disease (Betarbet *et al.*, 2000; Sherer *et al.*, 2003). Rotenone's advantages of being able to create an animal model exhibiting a slow progression of disease and the formation of LB-like structures outweighed the use of the well-documented but more acute neurotoxins 6-OHDA and MPTP which did not show any LB formation.

Ultimately, for application of an animal model as a tool for the development of systems PK-PD disease progression models, in which time-dependent changes in the biological system-specific parameters of diseased animals can be obtained (Post *et al.*, 2005), induction of a progressive form of the disease is important.

In the investigations described in **Chapter 4** the subcutaneous route of administration of rotenone was compared with intracerebral administration, directly into the median forebrain bundle (MFB), on parameters such as bodyweight and BBB permeability using sodium fluorescein as a marker and intracerebral microdialysis to quantify flurescein brain distribution. In addition, behavioural assessments were conducted using the rotorod for the subcutaneous model and amphetamine-induced rotometry for the intracerebral model. Post-mortem analysis consisted of assessing nigrostriatal damage based on immunohistological staining with TH and α-synuclein inclusions and peripheral organ pathology.

The results indicated that rotenone infused intracerebrally at the highest dose tested is able to create a progressive rat model for Parkinson's disease, which makes this model useful for research on PK-PD relationships at different stages of Parkinson's disease. The subcutaneous route of administration was found to be of limited value due to the occurrence of peripheral organ toxicity, which indirectly influenced the BBB permeability.

Research on the disposition and brain distribution of L-DOPA in the rotenone model of Parkinson's disease

Chapter 5 describes the research on the disposition and brain distribution of L-DOPA in the Rotenone model of Parkinson's disease.

Parkinson's disease treatment is still mainly focussed on symptomatic treatment by replacing the striatal dopamine by drugs like L-DOPA (Factor, 2008; Nyholm, 2006; Schapira, 2008). L-DOPA is actively transported across the BBB to the brain by the LAT-1 transporter and there are a number of indications on changes in BBB

transport of L-DOPA in animals (Carvey *et al.*, 2005) and in patients (Bartels *et al.*, 2008). In **Chapter 5**, we describe the investigation on potential changes in BBB transport of L-DOPA in conjunction with its brain conversion to the dopamine metabolites DOPAC and HVA, as a consequence of the disease using the intracerebral rotenone model as the rat model for Parkinson's disease. At 14 days after an unilateral injection of rotenone (5 μg) in Lewis rats, intracerebral microdialysis was used to measure endogenous and exogenous $brain_{ECF}$ concentrations of L-DOPA and $brain_{ECF}$ concentrations of DOPAC and HVA following different dosages of L-DOPA (10, 25 or 50 mg/kg). These measurements were used for the investigation of the relationship between plasma and $brain_{ECF}$ kinetics of L-DOPA, and the dopamine metabolites DOPAC and HVA in the untreated as well as in the treated brain side. Post-mortem analysis using striatal TH staining was used to determine "responders" to rotenone. Non-linear Mixed Effects Modeling (NONMEM) was used to develop a population based PK model of L-DOPA and metabolites. The results indicated that the disease conditions at 2 weeks post-rotenone-injection in the MFB did not result in any change in the kinetics (including the brain distribution) of L-DOPA. Merely, a clear effect of disease on the concentrations and elimination rates of DOPAC and HVA in brain was found, providing indirect information on decreased dopamine concentrations at the diseased brain side based on "formation-rate limited elimination" pharmacokinetic principles.

Summary, conclusions and perspectives

Finally, in **Chapter 6** the results presented in this thesis are discussed and perspectives for future research are presented.

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Chapter 2

Understanding Drug Response in Parkinson's Disease

The Role of the Blood-Brain Barrier

Paulien G.M. Ravenstijn, Meindert Danhof and Elizabeth C.M. de Lange

Abstract

Symptomatic treatment of the later stages of Parkinson's disease is far from optimal, while drugs that halt disease progression are not, yet, available. To improve drug treatment and drug development, detailed information on the interrelationship between disease, pharmacokinetics and drug response is needed. This review aims at the understanding of the mechanisms that govern drug response in Parkinson's disease. Special emphasis is on the impact of the BBB on drug response, specifically under diseased conditions like Parkinson's disease. It is concluded that systems pharmacology approaches are needed to investigate and understand the various mechanisms that determine the drug response as well as their interplay.

1. Introduction

Parkinson's disease is characterised by the loss of dopamine producing neurons in the striatum and substantia nigra pars compacta (SNc), resulting in clinical symptoms like bradykinesia, resting tremors, rigidity and postural instabilities (Thomas and Beal, 2007). Treatment of these symptoms of Parkinson's disease is still by replacing the lost brain dopamine. L-3,4-dihydroxyphenylalanine (L-DOPA) in combination with an aromatic amino acid decarboxylase (AAAD) inhibitor is currently still the golden standard. At later stages of the disease, when L-DOPA-related motor complications arise, dopamine agonists may be added to the treatment (Mercuri and Bernardi, 2005). The future for new drug therapies in Parkinson's disease is heading towards neuroprotection or neurorescue (Bonuccelli and Del Dotto, 2006; Chen and Le, 2006; Schapira, 2008b) as L-DOPA or dopamine agonists merely give relief to symptoms thereby increasing the quality of life, but are not halting or reversing the progress of Parkinson's disease. In most neuropharmacological studies the effects of drugs in the CNS are still related to the dose (Girgin *et al.*, 2008; Homma *et al.*, 2008; Kinon *et al.*, 2008; LeWitt *et al.*, 2008; Mischoulon *et al.*, 2008; Stern *et al.*, 2004; Walsh *et al.*, 2008). However, a dose-effect relationship is not informative as there are many mechanisms (e.g. plasma protein binding, blood-brain barrier (BBB) transport, within brain distribution, target interaction) that affect the brain distribution kinetics of the CNS drug and ultimately the drug response. Differences in factors such as genetics, species, gender, age, environmental and pathological conditions can influence these individual mechanisms and should therefore also be taken into consideration when comparing or making predictions on drug response. This

review gives a summary of the mechanisms involved in CNS drug response and the factors that may influence them and thereby the drug response. Special emphasis is on the BBB as a keyplayer in CNS drug response, and how the BBB functionality may change under diseased conditions like Parkinson's disease.

2. Parkinson's Disease

Disease characteristics

Parkinson's disease is the second most common progressive movement disorder (Dauer and Przedborski, 2003). The disorder affects several regions of the brain. One of these areas is the SNc that controls balance and movement. Clinical manifestations are a result of the loss of 50-80% of neuromelanin containing dopaminergic neurons in the SNc (and striatum) and include motor impairments such as resting tremor, bradykinesia, rigidity, gait difficulty and postural instability as well as non-motor symptoms (Dauer and Przedborski, 2003; Forno, 1996; Thomas and Beal, 2007). Non-motor symptoms include depression, constipation, pain, genitourinary problems, and sleep disorders and dominate the clinical picture of advanced Parkinson's disease. They contribute to severe disability, impaired quality of life, and shortened life expectancy (Chaudhuri *et al*., 2006). Residual nigral neurones show characteristic, eosinophilic inclusions called Lewy Bodies (LB) that are made up of neurofilaments and show ubiquitin immunoreactivity (McNaught *et al*., 2001). Other affected brain areas include regions of the brain that regulate involuntary functions such as blood pressure and heart activity. A schematic overview of the neuronal interconnections in Parkinson's disease as compared to the "healthy" situation is depicted in Figure 1.
Dopamine is the predominant catecholamine neurotransmitter in the mammalian brain, where it controls a variety of functions including locomotor activity, cognition, emotion, positive reinforcement, food intake, and endocrine regulation. Pathological conditions such as Parkinson's disease are linked to a dysregulation of dopaminergic transmission. (Missale *et al*., 1998).
Dopamine is the primary endogenous ligand for the G-protein-coupled dopamine receptors. These dopamine receptors can be divided into D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4). Through their different G-protein coupling, D1-like and D2-like receptors have opposing effects on adenylyl cyclase activity and cyclic AMP concentration. All five subtypes of dopamine receptors are found in the striatum (Smith and Kieval, 2000). The highest expression of both the D1 and D2 mRNAs are localised to the dorsal and ventral striatum as measured in normal human post mortem brains. Cortical expression is moderate

for D1 mRNA and very low for D2 mRNA. The SNc expresses D2 but not D1 mRNA. The D3 receptor has a specific distribution to limbic-related ventral striatum (Hurd *et al.*, 2001; Smith and Kieval, 2000). Low levels of the D4 receptor mRNA have been found in the basal ganglia. In contrast, this receptor appears to be highly expressed in the frontal cortex, amygdala, hippocampus, hypothalamus, and mesencephalon. The expression of the D5 receptor compared to the D1 receptor as measured in rat brain is very poor. The D5 receptor can be found in the cerebral cortex, lateral thalamus, diagonal band area, striatum, and, to a lesser extent, SNc, medial thalamus, and hippocampus (Hurd *et al.*, 2001).

The degree of forward locomotion is primarily controlled by the ventral striatum through activation of D1, D2, and D3 receptors. Activation of D2 autoreceptors, which results in decreased dopamine release, has been shown to decrease locomotor activity, whereas activation of postsynaptic D2 receptors slightly increases locomotion. Activation of D1 receptors has little or no effect on locomotor activity. However, there is synergistic interaction between D1 and D2 receptors in determining forward locomotion so that concomitant stimulation of D1 receptors is essential for D2 agonists to produce maximal locomotor stimulation. The D3 receptor, which has been shown to be mainly postsynaptically located in the nucleus accumbens, seems to play an inhibitory role on locomotion (Missale *et al.*, 1998). The synergistic interaction between D1 and D2 receptors is due to two main pathways in the nigrostratial tract. Both pathways receive a glutamatergic corticostriatal input (Gerfen, 2003). One pathway leads directly from the putamen to the Gpi (Figure 1, left panel). It has dopamine D1 receptors, co-express the peptides substance-P, and dynorphin and establish a monosynaptic inhibitory connection with GPi neurons. Neurons in the indirect pathway express the dopamine D2 receptor, which is coupled to the inhibitory Gi G-protein, as well as the A2A adenosine receptor, which is coupled to the stimulatory Golf G-protein. These neurons also co-express enkephalin (Gerfen, 2003). They project to the GPe which in turn influence the GPi by a monosynaptic inhibitory connection and indirectly through the GPe-STN-GPi projection (Obeso *et al.*, 2008). Thus, the direct and indirect pathways have opposing effects on the output function of the basal ganglia. Dopamine modulates glutamatergic effects on corticostriatal inputs by exerting a dual effect on striatal neurons: exciting Dl-receptor-expressing neurons in the direct pathway and inhibiting D2-receptor-expressing neurons in the indirect pathway (Obeso *et al.*, 2008).

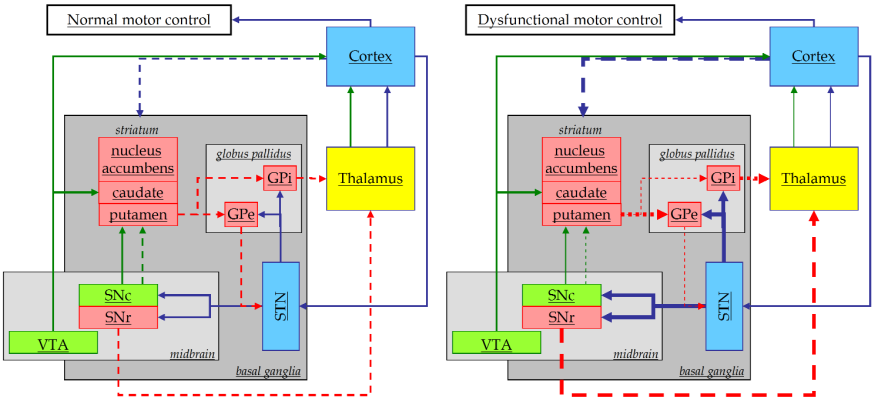

Figure 1: *Schematic overview of the neuronal interconnections involved in Parkinson's disease; (left) is the case of normal motor function and (right) is in the case of a dysfunctional motor control as seen in Parkinson's disease. The solid arrows depict excitatory projections and the dotted arrows depict inhibitory projections. The thickness of the arrows indicates the degree of activation of each projection (right). The red boxes and projections are GABAergic, the green boxes and projections are dopaminergic and the blue boxes and projections are glutamatergic. SNc: substantia nigra pars compacta; SNr: substantia nigra pars reticulata; VTA: ventral tegmental area; STN: subthalamic nucleus; GPe:globus pallidus, external segment; GPi: globus pallidus, internal segment. For the purpose of clarity, the neuroanatomy and interconnections are incomplete (Obeso et al., 2008; Smith et al., 2000; Smith et al., 2008)*

In Parkinson's disease, the direct striatal output tract from the striatum to the SNc is less active, as a result of decreased dopaminergic inhibitory tone. A study in MPTP (1-methyl-4-phenyl-1,2,3,6-tertahydropyridine)-lesioned mice also indicated a hyperactivity of the glutamatergic cortico-striatal pathway as a consequence of dopaminergic denervation resulting in an increase of striatal GABA levels (Chassain *et al.*, 2008). This leads to overactivity of the indirect striatal GABAergic output from the striatum to the GPe (Chassain *et al.*, 2008), diminishing inhibitory signals from the GPe to the STN and consequently resulting in excitatory projections to the GPi and SNc (Figure 1). These two basal ganglia nuclei, in turn, send inhibitory projections to the thalamus. Inhibition of the thalamus leads to decreased excitatory projections to the motor cortex, resulting in Parkinsonism (Del Tredici *et al.*, 2002). An increase in striatal glutamine concentrations was also seen in MPTP-lesioned mice, which might be a

strategy to protect neurons from glutamate excitotoxic injury after striatal dopamine depletion (Chassain *et al.*, 2008), although these results could not be confirmed in a study in 6-hydroxydopamine-lesioned (6-OHDA) rats (Kickler *et al.*, 2009). Excitotoxicity will be discussed in more detail later in this review.
Degeneration of the dopamine input to the striatum results in opposing affects in the direct and indirect pathways. The resulting functional imbalance is thought to be responsible for the bradykinesia of Parkinson's disease, which may be temporarily normalised by dopamine replacement therapy. However, direct striatal projection neurons become irreversibly supersensitive to D1 dopamine receptor activation, despite the fact that there is an actual decrease in receptor number. Recent studies show that this D1-supersensitive response results from a switch in D1-receptor-mediated regulation of protein kinase systems responsible for neuronal plasticity and is suggested to underlie dyskinesia produced by L DOPA treatment of Parkinson's disease (Gerfen, 2003).

Diagnosis & Biomarkers
Currently, the diagnosis of Parkinson's disease is based on patient history and physical examination alone as there is no simple and effective biomarker available (Savitt *et al.*, 2006). Using the current diagnostic criteria, 90% may be the highest accuracy that can be expected (Hughes *et al.*, 2001). Biomarkers might be used to assist in the diagnosis of Parkinson's disease. The search for suitable biomarkers has led to various studies using imaging techniques such as positron emission tomography (PET) and single photon emission computed tomography (SPECT). However, such studies provide markers of nerve terminal function rather than cell density and thus, like clinical assessments, are potentially influenced by compensatory mechanisms and direct effects of medication (Brooks, 2004). In post-mortem investigations and in animal research, tyrosine hydroxylase (TH) immunostaining is used as a golden standard to determine the degree of lesion in the dopaminergic neurons in striatum or SNc. However, this analysis can only be performed post-mortem. Availability of relevant biomarkers which could be measured in vivo, could result in a more detailed and mechanistic description of Parkinson's disease progression and serve as useful tools in disease models. A combination of biomarkers might be needed to provide a complete characterisation of treatment effects (beneficial and harmful) or disease progression (Lesko and Atkinson, Jr., 2001).
Other biomarkers for Parkinson's disease include clinical tests, blood tests, cerebrospinal fluid (CSF) tests and genetic tests. An example of a clinical test is the

pharmacological challenge in which the response to L-DOPA or a dopamine agonists is examined to differentiate Parkinson's disease from other parkinsonian syndromes (Dorsey *et al.*, 2006). Blood and CSF tests mainly focus on markers of oxidative stress such as the mitochondrial complex I level in blood or 8-hydroxy-2'-deoxy-guanosine, which results from oxidised DNA among others in blood and CSF (Michell *et al.*, 2004). Alternative blood markers which are potential biomarkers for Parkinson's disease are platelet α-synuclein, platelet monoamine-oxidase-B (MAO-B) activity and dopamine transporter density on peripheral lymphocytes (Dorsey *et al.*, 2006; Michell *et al.*, 2004). For the purpose of early clinical evaluation and effective patient management, diagnostic strategies based on the usual screening panels for nigrostriatal somatomotor symptoms associated with Parkinson's disease might be supplemented for persons at risk by tests designed to elicit signs such as symptoms related to dysautonomia, signs indicating subtle disruptions within the gain setting nuclei of the lower brainstem or quantifiable deficits in olfactory acuity, differentiation, or memory of substances smelled (Del Tredici *et al.*, 2002).

For the majority of the patients with Parkinson's disease, genetic factors do not clearly play a role, however for individuals with a family history of the disease, genetic testing can indicate the risk. Genetic testing can also be helpful in diagnosis as many cases of 'genetic' Parkinson's disease have a different clinical course than sporadic Parkinson's disease (Dorsey *et al.*, 2006).

Etiopathogenesis

Parkinson's Diseases often begins after the age of 50 (late onset), but may also begin before the age of 50 (early onset) or even before the age of 20 (juvenile onset). Parkinson's disease occurs in 1-2 % of individuals over 50 and in more than 3% of individuals above 75 years of age (Pankratz and Foroud, 2007). The prevalence of Parkinson's disease is higher in men than women (Van Den Eeden *et al.*, 2003). Estrogen has an effect on dopamine neurotransmission i.e. inhibition of dopamine reuptake, effect on dopamine synthesis and release and estrogen has shown to protect nigrostriatal neurons from toxic influences (Shulman, 2007) which might explain the gender difference in prevalence. An advancing age is associated with a faster rate of motor progression, decreased L-DOPA responsiveness, more severe gait and postural impairment, and more severe cognitive impairment and the development of dementia in patients with PD (Levy, 2007).

Risk factors

For Parkinson's disease, no single causative factor has been identified. Instead, various mechanisms- including mitochondrial defects, oxidative stress, excitotoxicity, genetic factors and apoptosis- seem to play a role. This suggests that the etiopathogenesis of Parkinson's disease is most likely multi-factorial. Certain risk and protective factors have been identified for Parkinson's disease. Smoking and (moderate) consumption of coffee or tea (caffeine intake) have been associated with a decreased risk for Parkinson's disease (Ascherio *et al.*, 2001; Hong *et al.*, 2008; Kandinov *et al.*, 2008; Morozova *et al.*, 2008; Saaksjarvi *et al.*, 2008). Hydroquinone and nicotine, two components of cigarette smoke apparently inhibit α-synuclein fibrillation and stabilize soluble oligomeric forms (Hong *et al.*, 2008). Caffeine or its metabolite paraxanthine might be neuroprotective via antagonist action on the adenosine A2 (A2A) receptor (Guerreiro *et al.*, 2008). Animal studies suggest that caffeine may protect the BBB against experimental parkinsonism (Chen *et al.*, 2008). Animal studies (McGeer and McGeer, 2004b) also suggest that nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the incidence of Parkinson's disease, but epidemiologic studies are inconclusive (Etminan and Suissa, 2006; Hancock *et al.*, 2007; Hernan *et al.*, 2006; Powers *et al.*, 2008; Ton *et al.*, 2006; Wahner *et al.*, 2007). Furthermore, rural living, farming, gardening and drinking well water have been identified as risk factors as these factors can be associated with exposure to pesticides (Chade *et al.*, 2006; Kamel *et al.*, 2007).

Toxic substances, in general, can cause neurological damage as was first demonstrated by MPTP which is associated with parkinsonism in young drug addicts (Langston *et al.*, 1983). MPTP, which is a pro-toxin, rapidly crosses the BBB and is converted to 1-methyl-4-phenylpyridinium (MPP+) which is transported into dopamine neurons where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain (Vila and Przedborski, 2003). Pesticides and metals are also named as neurotoxins involved in Parkinson's disease. Metals (eg iron and copper) have been investigated as potential risk factors on the basis of their accumulation in the SNc and their participation in harmful oxidative reactions such as the production of hydrogen peroxide during the oxidation of dopamine and the conversion of hydrogen peroxide to hydroxyl radicals which is catalysed by iron (Di Monte, 2003; Landrigan *et al.*, 2005). Manganese has been mentioned as potential risk factor (Landrigan *et al.*, 2005), however, it damages the globus pallidus and not the SNc. There is also an absence of nigral LBs and this is inconsistant with the neurological symptoms of Parkinson's disease (Perl and

Olanow, 2007). The widely used pesticide rotenone is, like MPTP, an inhibitor of complex I and able to induce the major features of Parkinson's disease in rats (Betarbet *et al.*, 2000; Ravenstijn *et al.*, 2008). Rotenone is able to cross cell membranes and is therefore likely to affect all cells but mainly targets dopaminergic neurons probably because dopamine metabolism is responsible for high basal levels of oxidative stress in the SNc (Giasson and Lee, 2000; Jenner, 2003; Mandemakers *et al.*, 2007). Epidemiological studies have revealed a relationship between pesticide exposure and a high risk for Parkinson's disease (Di Monte, 2003).

Mitochondrial dysfuntion & Oxidative stress

Molecular studies in neurotoxin based and genetic-based animal models suggest a major etiologic role for mitochondrial dysfunction in the pathogenesis of Parkinson's disease. Neurons in general appear to be more sensitive than other cells to mutations in genes, such as Opa1, Mfn1 and Dnm1l encoding mitochondrial proteins involved in the dynamic morphological alterations and subcellular trafficking of mitochondria (Mandemakers *et al.*, 2007). Post-mortem biochemical studies in patients have revealed mitochondrial defect (30-40% in complex I activity) in specifically the SNc of the brain and in platelets and muscle (Olanow and Tatton, 1999; Schapira, 2006) and animal studies in mice which have a knockout of mitochondrial transcription factor A in dopaminergic cells, have shown Parkinson-like symptoms (Onyango, 2008). Oxidative stress is linked to mitochondrial dysfunction as well as other components of the degenerative process such as excitotoxicity and inflammation (Schapira, 2008a). Oxidant stress and consequent cell death could develop in the SNc under circumstances in which there is (1) increased dopamine turnover, resulting in excess peroxide formation; (2) a deficiency in glutathione (GSH), thereby diminishing the brain's capacity to clear H_2O_2; or (3) an increase in active iron, which can promote OH· formation (Olanow and Tatton, 1999). The evidence for oxidative stress in Parkinson's disease has been summarised elsewhere (Jenner, 1991) and will not be repeated here. Complex I deficiencies in SNc mitochondria evoke free radical generation, which, in turn, impairs the function of the respiratory chain. Mitochondrial abnormalities decrease the activity of the ubiquitin proteasomal system (UPS), a process that is further exacerbated by the increased substrate load of oxidised protein from oxidative stress. Abnormalities in protein phosphorylation might influence the UPS and mitochondrial function.

Inflammation

Chronic inflammation may play an important role, if secondary, in the pathogenesis of Parkinson's disease (Hirsch *et al*., 2005; McGeer and McGeer, 2004a). The proinflammatory cytokine tumor necrosis factor-α (TNF-α) kills dopamine neurons *in vitro* and is elevated in the brains of patients with Parkinson's disease (Logroscino, 2005). The TNF-308A allele was found significantly more frequent in early onset patients compared to the controls and might influence the risk for the development and/or onset of Parkinson's disease (Bialecka *et al*., 2008a). Inflammation will enhance free-radical production including nitric oxide and peroxynitrite. It has recently been proposed that Parkinson's disease might be an autoimmune disease as a disruption of the BBB, as explained later, might result in the entry of immune cells leading to a progressive degenerative process (Monahan *et al.*, 2008).

Excitotoxicity

Free radical species will also be enhanced by excitotoxicty, which leads to nitric-oxide-mediated damage to the mitochondria (Schapira, 2008a). Excitotoxicity is an established cause of neurodegeneration that has been implicated in Parkinson's disease based on two possible mechanisms. The first is direct excitotoxicity resulting from increased glutamate formation. SNc dopaminergic neurons are rich in glutamate receptors and receive extensive glutamate innervation from the subthalamic nucleus (STN; Figure 1). Dopamine lesions disinhibit the STN which results in an increased firing rate of its output neurons leading to excessive calcium influx into the cell and nitric oxide (NO) formation (Figure 2). The second mechanism involves indirect excitotoxicity where a defect in mitochondrial function results in the loss of the ATP-dependent Mg-blockade of N-methyl-D-aspartate (NMDA) receptors leading to a calcium influx into the cell under physiological glutamate levels (Figure 2) (Olanow and Tatton, 1999). NO reacts with superoxide radical to form peroxynitrite and hydroxyl radical which are both powerful oxidizing agents (Beckman *et al*., 1990). Also the mitochondrial respiratory chain might, in turn, be damaged by sustained exposure to NO (Bolanos *et al*., 1996). Next to NO formation, the formation of L-ornithine decarboxylase (ODC) is increased due to excessive calcium influx. ODC is an enzyme involved in the synthesis of polyamines (spermidine, spermine and putrescine) (Williams, 1997). Polyamines may in turn stimulate the NMDA receptor and result in a 'run-away-process'. Cell death in Parkinson's disease occurs by way of apoptosis rather than necrosis (Olanow and Tatton, 1999).

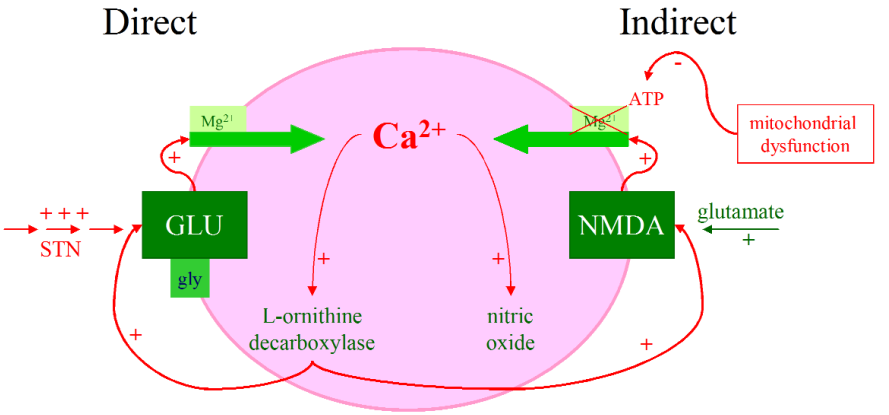

Figure 2: *Mechanisms of excitotoxicity STN: Subthalamic Nucleus; GLU: Glutamate; gly: glycine; NMDA: N-methyl-D-aspartate*

Neuronal apoptosis can be induced by low concentrations of toxins or substances such as L-DOPA, dopamine, iron, MPTP among others (Tatton *et al.*, 1997).

Genetics

Most cases of Parkinson's disease (>95%) are sporadic, although some genes (associated with the PARK loci) have been identified and linked to rare forms of Parkinson's disease. Among them are the SNCA or α-synuclein (PARK1), LRRK2 (PARK8), which result in autosomal dominant Parkinson's disease; PRKN or Parkin (PARK2), DJ-1 (PARK7), PINK1(PARK6), which result in autosomal recessive Parkinson's disease (Pankratz and Foroud, 2007); UCHL1 (PARK5), which has been implicated but not confirmed and a link with the locus PARK3 has been identified but no gene has been found. So there are only five clearly defined genetic causes of Parkinson's disease. A more detailed description of the consequences of these mutations are described elsewhere (Farrer, 2006; Pankratz and Foroud, 2007; Thomas and Beal, 2007).

3. Current Drug Treatment of Parkinson's Disease

Symptomatic treatments

Treatment of the symptoms of Parkinson's disease is currently by replacing the lost brain dopamine. Unfortately these therapies only provide temporary relief from early symptoms and do not halt disease progression. Moreover, pathological changes outside of the motor system leading to cognitive, autonomic, and

psychiatric symptoms are not sufficiently treated by current therapies (Savitt *et al.*, 2006).

The most widely used drug in the treatment of Parkinson's disease is L-DOPA (Kostrzewa *et al.*, 2005; Mercuri and Bernardi, 2005). L-DOPA is metabolised by AAAD to dopamine. AAAD is present in low concentrations in most body tissues and in high concentrations in liver, kidney, intestinal mucosa and plasma (Leppert *et al.*, 1988). A second but less important biotransformation pathway is the O-methylation of L-DOPA to 3-O-methyldopa (3-OMD) by COMT (Nutt and Fellman, 1984). Carbidopa and benserazide are the two most commonly used decarboxylase inhibitors used in combination with L-DOPA, increasing the proportion of L-DOPA dose in plasma (increase of C_{max} and AUC, decrease of t_{max}) in such a manner that a 70 to 80% reduction in total daily dose of L-DOPA is possible in order to obtain the same clinical benefits (Cedarbaum, 1987). The rate of gastric emptying is the principle determinant in the disposition of L-DOPA. Absorption of L-DOPA in the small intestine occurs by means of an active, saturable, large neutral amino acid carrier system (Deleu *et al.*, 2002). L-DOPA reaches its effect site by crossing the BBB via an active transporter, the large amino acid transporter (LAT) (del Amo *et al.*, 2008). In brain tissue, L-DOPA is metabolised for about 69% by AAAD and for about 10% by COMT (Nutt and Fellman, 1984).

During early Parkinson's disease, the effect of L-DOPA is long lasting and stable. However, at later stage of the disease and after long-term L-DOPA treatment, motor complications may develop, starting with "wearing-off" which is the progressive shortening of the effect of L-DOPA to 4 hours or less after administration of the dose. In the more advanced stages of the disease, the "on-off" phenomenon appears, in which the effect of L-DOPA can suddenly and unpredictably disappear, resulting in a mobile ("on") patient to become immobile ("off") (Nyholm, 2006; Bhidayasiri and Truong, 2008). At the same time, patients may develop dyskinesias (involuntary movements), which are considered to be related to large and multiple doses of L-DOPA (Bhidayasiri and Truong, 2008; Obeso *et al.*, 2004). The underlying pathology of motor complications is believed to be due to the progressive loss of dopaminergic neurons that results in a decreased ability to buffer fluctuations in dopamine levels in the brain, coupled with the short half-life of L-DOPA of approximately 1 hour (Deogaonkar and Subramanian, 2005; Tse, 2006). Animal studies as well as a few human studies have revealed that motor complications developed after intermittent

administration of L-DOPA but did not develop when L-DOPA was given continuously (Nyholm, 2006; Olanow *et al.*, 2004; Stocchi, 2005). The high degree of interindividual variability in absorption after oral administration is significantly reduced when L-DOPA is given intraduodenally or intraperitoneally (Bredberg *et al.*, 1994). Consequently, to overcome these motor complications, L-DOPA can be given with a shorter dosing interval i.e. in a more continuous manner (Stocchi, 2005), but also by administration of L-DOPA in combination with a COMT inhibitor or a dopamine agonist. Also, L-DOPA may be administered together with a MAO-B inhibitor, such as selegiline or rasagiline, which inhibits dopamine metabolism (Bhidayasiri and Truong, 2008; Factor, 2008; Nyholm, 2006; Nyholm, 2006; Stacy and Galbreath, 2008). The symptomatic effects of selegiline were thought not only to relate to MAO inhibition but were also thought to be associated with an amphetamine effect (enhancing release of dopamine) as selegiline is metabolised to L-amphetamine via the first pass in the liver (Factor, 2008).

Concomitant medication with a COMT inhibitor, such as entacapone or tolcapone increases the amount of L-DOPA available for transport across the BBB as well as reduces the amount of 3-OMD which is a competitor to L-DOPA in the uptake by the LAT transporter at the BBB (Wade and Katzman, 1975). Entacapone is a mainly peripherally acting COMT inhibitor and has a low BBB penetration. Tolcapone, however, is able to cross the BBB and also act on central COMT (Ceravolo *et al.*, 2002; Kaakkola and Wurtman, 1992; Napolitano *et al.*, 2003; Russ *et al.*, 1999), although it has not been investigated whether this further enhances the efficacy of L-DOPA (Forsberg *et al.*, 2003). Tolcapone increases the AUC and C_{max} of L-DOPA but does not influence its PK-PD relationship, making it a suitable add-on to L-DOPA therapy (Baas *et al.*, 2001). L-DOPA treatment has been shown to increase plasma homocysteine levels in Parkinson's disease patients as it is metabolised via O-methylation by COMT using S-adenosyl-L-methionine (SAM) as the methyl donor which results in the subsequent formation of homocysteine (L-DOPA induced hyperhomocysteinaemia). Increased levels of homocysteine might lead to an increased risk for coronary arterial diseases. Based on studies using rats and confirmed in human studies, entacapone may reduce L-DOPA-induced hyperhomocysteinaemia in patients with Parkinson's disease (Nissinen *et al.*, 2005; Valkovic *et al.*, 2005) and thereby reducing the risk of pathologies probably linked to it.

Dopamine agonists, such as ropinerole, apomorphine, bromocriptine, pergolide, cabergoline, lisuride or pramipexole are less effective than L-DOPA in yielding

symptomatic relief and almost all patients will require L-DOPA at some point. Dopamine agonists are drugs acting directly to stimulate dopamine receptors, classified as D1-like (D1, D5) and D2-like (D2, D3, D4). Postsynaptic D2 receptor stimulation is closely associated with antiparkinsonian activity and presynaptic D2 receptor stimulation may have neuroprotective effects. Optimal therapeutic response is thought to require stimulation of both D1 and D2 receptors (Deleu *et al.*, 2002). Dopamine agonists have a reduced risk of the development of dyskinesias which is probably related to the longer $t_{1/2}$ relative to L-DOPA (Savitt *et al.*, 2006; Yamamoto and Schapira, 2008).

Future symptomatic therapies which are currently being investigated for the treatment of Parkinson's disease include non-dopaminergic agents such as the adenosine A2A antagonists which are related to caffeine. The A2A receptors are co localised with D2 receptors on striatal medium spiny GABAergic neurons of the striatopallidal pathway, and antagonists of A2A receptors have been found to enhance the effects of dopamine at the D2 receptors on striatopallidal neurons, thus suppressing inhibitory GABAergic output and improving parkinsonian symptoms (Petzer *et al.*, 2009). Another way to counter the excessive excitatory stimulation or to treat the glutamate excitotoxicity, as described earlier, is by glutamate antagonist drugs such as amantadine and riluzole which are currently under investigation for the treatment of patients with motor fluctuations and dyskinesias (Factor, 2008; Wu and Frucht, 2005). Finally, noradrenergic drugs are being examined, because this neurotransmitter appears to influence motor and affective symptoms.

Neuroprotective treatments

The most important goal for drug development in Parkinson's disease is neuroprotection or neurorescue (Bonuccelli and Del Dotto, 2006; Chen and Le, 2006; Schapira, 2008b). Some of the drugs already in use as treatment in Parkinson's disease or currently under investigation seem to possess neuroprotective properties

Dopamine agonists such as bromocriptine, pergolide, ropinirole and pramipexole have been shown to act as free radical scavengers against hydroxyl radicals, NO radicals and to have antioxidant effect (Deleu *et al.*, 2002). Pramipexole has been shown to reduce nigrostriatal cell death in MPTP-treated non-human primates (Iravani *et al.*, 2006). Furthermore, MAO-B inhibitors (selegiline, rasagiline) may also possess neuroprotective properties in part by reducing the damaging effect of dopamine turnover in the brain. These effects of MAO-B inhibitors are especially

relevant when considering that the brain shows an age-related increase in MAO-B activity (Petzer *et al.*, 2009). Next, as mentioned previously, caffeine consumption has been associated with a decreased risk for Parkinson's disease and it has been thought that it might be neuroprotective via antagonist action on the A2A receptor (Guerreiro *et al.*, 2008). This is further substantiated in a study in which caffeine was able to protect MPTP-induced BBB dysfunction in mice (Chen *et al.*, 2008). Another study revealed that caffeine and other A2A-antagonists were able to attenuate MPTP-induced loss of striatal dopamine and dopamine transporter binding sites in mice (Chen *et al.*, 2001).

Coenzyme Q10 is able to enhance mitochondrial function, increasing ATP production, and also functions as an antioxidant. A study in patients with early Parkinson's disease demonstrated that high doses of coenzyme Q10 were associated with a reduced rate of deterioration in motor function (Bonuccelli and Del Dotto, 2006). Diet supplement of creatine not only enhances mitochondrial function but also reduces oxidative stress through stabilisation of mitochondrial creatine kinase. Moreover, creatine supplementation may exert an anti-apoptotic effect because creatine kinase acts to inhibit opening of the mitochondrial transition pore and the consequent triggering of apoptosis. A few experimental studies suggest a neuroprotective role of dietary intake of creatine in Parkinson's disease models (Matthews *et al.*, 1999). In the MPTP model of Parkinson's disease, amantadine, a NMDA receptor antagonist, showed partial protection suggesting beneficial effects not only on the clinical features but also on disease progression (Rojas *et al.*, 1992). Memantine is another aminoadamantane derivative with weak NMDA receptor antagonistic properties. Preclinical data suggest that the potential neuroprotective effect of memantine might be mediated by the increase of endogenous production of brain cell-derived neurotropic factor (BDNF) in the brain (Bonuccelli and Del Dotto, 2006). Minocycline is an antimicrobial tetracycline compound which has shown in the MPTP and 6-OHDA rodent models of Parkinson's disease an enhanced survival of dopaminergic nigral neurons (McGeer and McGeer, 2007). This drug may act by inhibiting microglial activation. Other experimental data suggest that minocycline is able to block caspase 1 and 3 and to counteract apoptosis (Bonuccelli and Del Dotto, 2006). As mentioned above, animal studies (McGeer and McGeer, 2004b) suggest that NSAIDs decrease the incidence of Parkinson disease which gives it potential neuroprotective relevance. A more detailed understanding of neuroinflammatory mechanisms in Parkinson's disease will lead to new cellular and molecular targets. Future treatment may involve combination therapies with drugs directed

at both inflammatory and non-inflammatory mechanisms (Klegeris *et al.*, 2007).
In general, for major progress to be made in the treatment of Parkinson's disease, we need a paradigm shift away from focus on dopamine and dopaminergic neurons. With current medications and surgical options designed to restore brain dopaminergic function, we are close to going about as far as we can with this avenue of treatment and investigation. It has become increasingly clear that clues to the etiology of Parkinson's disease will not be found via studies of dopamine metabolism, and major treatment advances lie beyond the dopaminergic nigrostriatal system (Ahlskog, 2007). Understanding why and how susceptible cells in motor and non-motor regions of the brain die in Parkinson's disease is the first step toward preventing this cell death and curing or slowing the disease (Savitt *et al.*, 2006).

4. Mechanisms Involved in Target Site Distribution of CNS Drugs

In most neuropharmacological studies the effect of drugs in the CNS are still related to the dose (Girgin *et al.*, 2008; Homma *et al.*, 2008; Kinon *et al.*, 2008; LeWitt *et al.*, 2008; Mischoulon *et al.*, 2008; Stern *et al.*, 2004; Walsh *et al.*, 2008). However, a single unique dose-CNS response relationship does not exist. CNS target site distribution kinetics is determined by many mechanisms such as plasma protein binding, BBB transport and within brain distribution. The ultimate kinetics of the drug at the target will, combined with target interaction and signal transduction, account for the CNS drug response profile. Here we discuss the main mechanisms that play a role in target site distribution of CNS drugs, together with some examples of conditions that influence the particular mechanism.

Plasma protein binding

After administration, drugs will enter the plasma compartment and may circulate either in the free form or associated with one or more binding sites, such as on plasma proteins. Plasma has two major proteins: albumin and α_1-acid glycoprotein, with different capacities and characteristics for drug binding (Kremer *et al.*, 1988), and other plasma constituent like lipoproteins, erythrocytes and α-, β-, γ-globulins (Wright *et al.*, 1996). Albumin is the most important drug-binding protein due to its high concentration in plasma. Albumin has several high- and low-affinity binding sites and is mainly involved in the binding of acidic drugs (Day and Myszka, 2003; Murai-Kushiya *et al.*, 1993; Notarianni, 1990; Piafsky, 1980). In contrast α_1-acid glycoprotein is mainly involved in the binding

of neutral and basic drugs (Kopecky, Jr. *et al.*, 2003; Kremer *et al.*, 1988). Especially α_1-acid glycoprotein is of interest as its levels are susceptible to changes.

It is well-known that the extent and strength to which drugs are bound to plasma components is important for the distribution of the drug over the body compartments (Rowland and Tozer, 1995) and will determine the time-dependent fraction of a drug that is available for transport into the brain (Fenstermacher *et al.*, 1995; Filippi and Rovaris, 2000). It has been suggested that only the free (unbound, dissociated) drug in plasma (Robinson and Rapoport, 1986; Rowley *et al.*, 1997) is available for transport across the blood to brain barriers and determines the intensity of the response for drugs such as benzodiazepines, opiates and steroids (Cox *et al.*, 1998; Kim *et al.*, 1997; Mandema and Danhof, 1992; Visser *et al.*, 2003a). However, some plasma protein bound drugs seem to cross the BBB (Cornford *et al.*, 1992; Jolliet *et al.*, 1997; Lin *et al.*, 1987; Lolin *et al.*, 1994; Urien *et al.*, 1987). For propanolol (Pardridge *et al.*, 1983; Pardridge, 1988b) it has been reported that the total rather than the free concentration determines the response, indicating that the bound drug is available for transport into the brain. Furthermore, it has been shown that drugs which bind fairly selectively to one of the main binding sites of albumin, Sudlow II (e.g., benzodiazepines and tryptophan) showed enhanced dissociation and a greater uptake into the brain than could be accounted for by the Kety-Crone-Renkin equation which is commonly used to analyse capillary transport (Fenerty and Lindup, 1989; Jones *et al.*, 1988; Lin and Lin, 1990; Tanaka and Mizojiri, 1999). However, other studies indicated no enhanced dissociation for drugs bound to Sudlow I (warfarin) or Sudlow II (ibuprofen) or both (tolbutamide and valproate) as measured in human, bovine and rat (Mandula *et al.*, 2006).

Although albumin levels are generally decreased in the elderly, changes in plasma protein binding in the elderly are generally not attributed to age, but rather to physiological and pathophysiological changes or disease states that may occur more frequently in the elderly and which most often affect the binding affinity (Grandison and Boudinot, 2000). Furthermore, pathological conditions may alter the plasma protein concentrations, thereby affecting the binding capacity. The α_1-acid glycoprotein is a major acute phase protein of which the concentration may rise in response to systemic tissue injury, inflammation or infection (Fournier *et al.*, 2000). Surgical procedures such as instrumenting rats with permanent blood cannulas will increase serum α_1-acid glycoprotein levels and binding (van Steeg *et al.*, 2007). Also, genetic variation can influence plasma protein concentrations,

as shown in a study on differences in the pharmacokinetics of indinavir and lopinavir for the various phenotypes of the α_1-acid glycoprotein (Colombo *et al.*, 2006). The impact of genetic variation, age and (patho)-physiological changes on drug response will be discussed elsewhere in this paper.

Cerebral blood flow

The cerebral blood flow determines the maximal rate at which the drug can be delivered to the brain, if not restricted by the transport across the blood brain barriers itself. In flow-limited transport conditions, alteration in blood flow thus has an impact on drug delivery to the brain. Alteration in cerebral blood flow may be the result of changes in two parameters: 1) changes in the linear velocity of blood flow through the perfused capillaries and 2) variations in the total number of the perfused capillaries in the brain ("effective perfusion") (Fenstermacher *et al.*, 1995). When the linear velocity of blood flow is increased, the diffusional influxes of highly permeable drugs across the BBB will increase (and vice versa), while BBB transport of slightly or virtually impermeable drugs will essentially be unchanged. Increase in linear velocity of blood flow may result from situations like hypercapnia, hypoxia, or may be induced by drugs such as nicotine. Also a decrease in local cerebral blood flow may be drug induced as has been reported for pentobarbital. Changes in the number of effectively perfused brain capillaries will change the surface accesible for transport across BBB, and therefore potentially affect blood-brain transport of all drugs. A small increase in the number of perfused capillaries has been reported for hypercapnia and upon administration of nicotine (Fenstermacher *et al.*, 1995).

The blood-brain barrier (BBB) and blood-CSF-barrier (BCSFB)

The brain is separated from direct contact with blood by two barriers. The first and largest barrier is the BBB. The BBB is mainly formed by brain capillary endothelial cells (BCEC) which distinguish themselves from peripheral endothelial cells by the presence of tight junctions, the lack of fenestrations, an increased mitochondrial content and a very low pinocytotic activity (Hawkins and Davis, 2005). Tight junctions prevent the transport of large hydrophylic compounds between blood and brain (Brightman and Reese, 1969; Huber *et al.*, 2001). The BBB is a highly dynamic barrier with its functionality being regulated by surrounding astrocytes, neurons, perivascular microglial cells and microvascular pericytes (Abbott, 2002; Bodor and Brewster, 1982; Cornford, 1985; Davson and Oldendorf, 1967; Hawkins and Davis, 2005; Hori *et al.*, 2004; Kim *et*

al., 2006; Pardridge, 1988b; Rubin and Staddon, 1999; Vorbrodt, 1988). The morphology of the BBB restricts free flow of compounds between blood and the brain making diffusion difficult for the required nutrients such as oxygen and glucose and other essential substrates to penetrate into the brain. However, a number of mechanisms and highly selective transporters on the membranes of the BCEC are involved in the influx and efflux of the required substances (Abbott and Romero, 1996; Kusuhara and Sugiyama, 2005; Ohtsuki, 2004; Tsuji, 2005). A

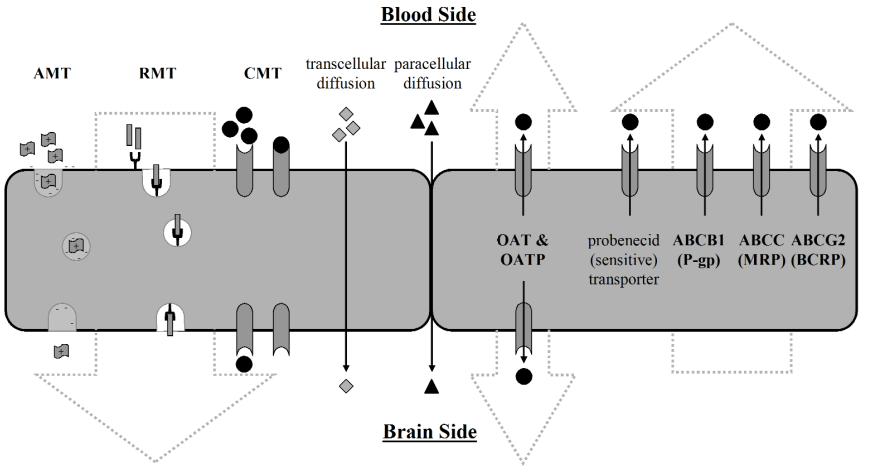

Figure 3: *Schematic overview of the influx (left side) and efflux (right side) transport mechanisms at the BBB. The OAT transporters are both inlfux and eflux transporters. This picture is adapted from Abbott and Romero (1996) and Kushuhara and Sugiyama (2005). AMT=absorptive mediated trancytosis; RMT=receptor mediated transport; CMT=carrier mediated transport; OAT= organic anion transporter; OATP= organic anion transporting polypeptide; P-gp=P-glycoprotein; MRP= multi-drug Resistance Protein; BCRP= Breast Cancer Related Protein.*

schematic overview of these transport mechanisms is depicted in Figure 3.

The second barrier is the BCSFB, which is a composite barrier made up of the choroid plexus epithelial cells, the arachnoid membrane and the circumventricular organs (such as the area postrema, median eminence, neurohypophysis and pinal gland) (de Lange, 2004). The choroid plexus is a leaf like structure that more or less floats in the brain ventricles. The BCSFB has fenestrated and, therefore, highly permeable capillaries. The barrier function of the BCSFB is provided by the tight junctions between the cells of the ependymal layer at the apical site, which contacts the CSF. These tight junctions are slightly more permeable than those of the BBB (Cserr, 1971; Meller, 1985; Milhorat, 1976;

Spector and Johanson, 1989). It should be noted, however, that in the circumventricular organs, the function and structure of the capillary endothelium is different (Gross *et al.*, 1986). In this small portion of the brain, the capillaries are fenestrated and permeable, for instance to serum proteins, thus in general have higher blood-to-tissue transport rates.

Passive transport

Solute molecules can cross membranes by the mechanisms of simple diffusion, either the paracellular or the transcellular route (Figure 3). The rate and extent of drug transport across the blood to brain barriers (Ghersi-Egea and Strazielle, 2001) is determined both by blood to brain barrier characteristics as well as by the physicochemical properties of the drug (Ghersi-Egea and Strazielle, 2001; Greig *et al.*, 1987; Groothuis and Levy, 1997; Gross *et al.*, 1986; Hammarlund-Udenaes *et al.*, 1997; Hammarlund-Udenaes, 2000; Hesselink *et al.*, 1999). Passive transport (diffusion) depends on size, charge at actual pH, and lipid solubility of the drug. Lipophilic, small, and non-charged drugs diffuse more easily across membrane (transcellularly) than hydrophilic, large and charged drugs (de Boer *et al.*, 2003). For drugs that easily cross the BBB, the cerebral blood flow may become the determinant in (mainly) the rate of transport across the barrier membranes. For the more hydrophilic drugs, paracellular diffusion becomes more important, which is restricted by the presence of the narrow tight junctions between the cells of the BBB and BCSFB. This makes that for paracellular diffusion the size of the drug relative to that of the space in the tight junctions is important (Groothuis and Levy, 1997; Levin, 1980; Oldendorf, 1970; Oldendorf, 1974).

Active transport

Transport across the BBB does not only occur on the basis of diffusion only. The brain endothelial cells and the choroid plexus epithelial cells express numerous influx and efflux transporters (Angeletti *et al.*, 1997; Bouw *et al.*, 2001b; Collins and Dedrick, 1983; Cordon-Cardo *et al.*, 1989; de Lange, 2004; Gao and Meier, 2001; Ghersi-Egea and Strazielle, 2001; Johnson *et al.*, 1993; Jolliet *et al.*, 1997; Loscher and Potschka, 2005; Nishino *et al.*, 1999; Ogawa *et al.*, 1994; Ooie *et al.*, 1997; Rao *et al.*, 1999; Schinkel *et al.*, 1994; Wijnholds *et al.*, 2000). Highly selective active transporters perform the influx of required nutrients and essential substrates and the efflux of waste and toxic product and can be divided into absorptive-mediated transcytosis (AMT), receptor-mediated transport (RMT) and carrier-mediated transport (CMT) (Figure 3). The AMT is based on the principle that polycations

interact specifically with negatively charged substances in the membrane of the endothelial cells. AMT mainly accounts for the transport of e.g. catonised albumins and histones (Alavijeh *et al.*, 2005; Bickel *et al.*, 2001). RMT transport large molecules like proteins and peptides which are required for metabolism processes in the brain. There are different types of RMT's at the BBB that have their own specific ligand such as the insulin receptor, the transferrin receptor or the leptin receptor. The RMT's transport the ligand into the cytosol through receptor mediated endocytosis (de Boer and Gaillard, 2007; Jones and Shusta, 2007). CMT's have the function to provide the brain with nutrients such as glucose (mainly by the GLUT-1 transporter (Pardridge *et al.*, 1990)), amino acids (e.g. Large Neutral Amino Acid -LNAA or LAT-transporter (Boado *et al.*, 1999)), purine bases, nucleosides, vitamins, hormones and others (Abbott and Romero, 1996). Other influx transporters include the nucleoside transporter system, the organic cation transport system (OCT), the organic anion transporter (OAT) and the organic anion transporting polypeptide (OATP) (Anzai *et al.*, 2006). The latter two systems (OAT and OATP) also act as efflux transporters. These transport systems may have a role in drug transport into the brain, although not for all transporters such a role has been clearly observed. For the LAT transport system, several amino-acid mimetic drugs like L-DOPA, α-methyl-dopa (Matsuo *et al.*, 2000; Wade and Katzman, 1975), α-methyl-trypsine, baclofen, gabapentin and phenylalanine mustard probably are substrates. Melphalan uptake into the brain is facilitated, by sharing the LAT system at the BBB (Greig *et al.*, 1987). Drugs bearing a monocarboxylic moiety such as simvastatin, lovastatin acid and pravastin, cross the BBB via the monocarboxylic acid transport systems (Tsuji, 2005). Pramipexole, a D2 receptor agonist used in the treatment of Parkinson's disease, is a cationic drug which not only crosses the BBB by diffusion but also via an (still to be identified) organic cation-sensitive transporter (Okura *et al.*, 2007).

Efflux transport

With regard to active drug transport, more is known about drug transport out of the brain by efflux transporters (Figure 3). Examples of such transporters are P-glycoprotein (P-gp or ABCB), the Multi-drug Resistance Protein family (MRP's or ABCC's (de Lange, 2007)) and the more recently discovered Breast Cancer Related Protein (BCRP, also known as ABCG2) (Cisternino *et al.*, 2004; Eisenblatter *et al.*, 2003). P-gp, MRP and BCRP are energy-dependent efflux pumps and belong to the ATP-binding cassette (ABC) transporter family.

P-gp is a broad spectrum efflux pump which can recognize and transport basic

and uncharged substrates in the size of 250Da -1850Da, as well as zwitterionic and positively charged substrates. It has been shown to be present at the luminal face (blood side) of the BBB (Cordon-Cardo *et al.*, 1989) and is also expressed at the choroid plexus (Rao *et al.*, 1999), however at these epithelial cells at the abluminal face (CSF side) then acting as an influx transporter. Substrates for both P-gp and MRP1 (see below) actually are cleared from the choroidal epithelial cells and may therefore protect these cells, thereby contributing to the detoxification of the choroid plexus itself (de Lange, 2004). P-gp may be inducible (Tatsuta *et al.*, 1994) and has many structurally diverse substrates (Seelig, 1998). Especially the use of mdr1a(-/-) mice (Schinkel *et al.*, 1994) has clarified the impact of this efflux transporter on brain concentrations of many drugs including dexamethasone, domperidone, indinavir, digoxin, vinblastin, morphine, sparfloxacin, amitryptiline, and cyclosporin A (de Lange *et al.*, 1998; de Lange *et al.*, 2000; Desrayaud *et al.*, 1998; Kim *et al.*, 1998; Mayer *et al.*, 1997; Meijer *et al.*, 1998; Schinkel *et al.*, 1995; Schinkel *et al.*, 1996; Uhr *et al.*, 2000; Xie *et al.*, 1999).

The influence of MRP transporters on BBB and BCSFB transport is dependent on their localisation at these membranes. Using primary cultured bovine brain microvessel endothelial cells (BBMEC) as a model for the BBB, Western blot analysis for MRPs and confocal laser scanning microscopy to determine membrane localisation of MRPs in BBMEC indicated that MRP1 and MRP5 are predominantly present at the apical plasma membrane and an almost equal distribution of MRP4 on the apical and basolateral plasma membrane of BBMEC. These orientations are different from those observed in polarised epithelial cells as model of the BCSFB (Zhang *et al.*, 2004). The use of mrp1(-/-) and mdr1ab(-/-) mice have shown the importance of MRP1 efflux transport at the level of the BCSFB in lowering the CSF concentrations of etoposide at least in the absence of P-gp for which it is also a substrate besides for MRP1 (Wijnholds *et al.*, 2000).

BCRP is a half transporter that displays drug resistance. BCRP shows clear similarity to P-gp in tissue distribution and to which substrates it binds. BCRP can actively transport a wide range of substrates ranging from chemotherapeutic agents to organic anion conjugates and also fluorescent compounds such as rhodamine 123 and Hoechst 33342 as well as chemical toxicants. BCRP is involved in multi drug resistance in cancer, especially with regard to acute myeloid leukemia. Also, it plays a role in the survival of stem cells under hypoxic conditions and might play a role in regulating stem-cell differentiation. BCRP is highly expressed at the luminal surface of the endothelial cells of human brain microvessels (de Lange, 2007; Mao and Unadkat, 2005).

The probenecid (sensitive) transporter is another efflux transporter at the BBB and BCSFB, involved in elimination of probenecid and a variety of other drugs. Mainly brain elimination is increased for a number of anti(retro)viral drugs (Groothuis and Levy, 1997; Takasawa *et al.*, 1997; Wong *et al.*, 1993) while increased efflux from CSF has been reported for penicilline and to a smaller extent other ß-lactam antibacterials like ampicilline, cefodizime cefotaxime and ceftriaxone (Dacey and Sande, 1974; Ogawa *et al.*, 1994; Spector, 1986; Spector, 1990), and methotrexate (Balis *et al.*, 2000). Also, after coadministration of probenecid, brain ECF concentrations of a few NMDA-receptor antagonists increased, and a prolongation of their anticonvulsant activity was found (Hesselink *et al.*, 1999).

As mentioned before, OATP and OAT also belong to the efflux transporters present at the BBB. However, OATPs and OATs are driven by a drug or ion gradient and are not energy-consuming. For further information about the active efflux transporters please see Anzai *et al.*, 2006; Borst *et al.*, 2000; Dallas *et al.*, 2006; Ebinger and Uhr, 2006; Kusuhara and Sugiyama, 2005; Loscher and Potschka, 2005; Schinkel, 1999; Sun *et al.*, 2003. The active-efflux transporters are multidrug resistant and limit the penetration of drugs into the brain and, next to metabolic degradation (Lee *et al.*, 2001), this can restrict its effect (Ebinger and Uhr, 2006; Groenendaal *et al.*, 2007b; Schinkel *et al.*, 1996). Bromocriptine, a D2 agonist used in the treatment of Parkinson's disease, has shown to be a substrate for P-gp (Vautier *et al.*, 2006). Changes on drug distribution into the brain for compounds are expected upon coadministration of other substrates or inhibitors of the same transporter, i.e. P-gp substrates or P-gp reversal agents or inhibitors (Klopman *et al.*, 1997; Mayer *et al.*, 1997). Several overviews of drugs which are substrates for the various efflux transporters have been published, for example by (Loscher and Potschka, 2005) and by (Anzai *et al.*, 2006).

Within brain distribution

A generalised physiological compartment model (Collins and Dedrick, 1983) shows the main factors that determine the entry into and distribution of drugs between brain extracellular fluid (brain ECF), intracellular brain compartments, ventricular and lumbar CSF. Thus, once within the brain, the kinetics of drug exchange between brain ECF, brain intracellular space (ICS) and CSF, as well as elimination by bulk flow/CSF turnover and potential local metabolic enzymes determine the time course of the concentration in specific parts of the brain (Mayer *et al.*, 1997)

ECF-CSF exchange

Diffusion between brain ECF and CSF is governed by the concentration gradient across the layer of ependymal cells that separates the brain ECF from the CSF (de Lange and Danhof, 2002; Mayhan, 2001). This layer possesses structural and enzymatic characteristics necessary for the clearance of a wide variety of substances in the CSF, thus forming a metabolic barrier at the brain ECF-CSF interface (Bruni, 1998; Del Bigio, 1995). Apart from that, concentrations in brain ECF and CSF may influence each other by diffusion (Fenstermacher *et al.*, 1974; Malhotra *et al.*, 1994). Diffusive transport will be more important in case of larger concentration gradients. In relative terms, after systemic administration of a drug, only small concentration gradients occur between brain ECF and CSF when transcapillary passage is rapid compared with CSF turnover (de Lange and Danhof, 2002; Meijer *et al.*, 1998), whereas the situation is reversed for drugs with permeability-limited BBB transport. For these compounds, significant concentration gradients between brain ECF and CSF exist after systemic administration (Agon *et al.*, 1991). On the other hand, following administration of a drug directly into the CSF, rapid BBB passage creates a steep gradient between brain ECF and CSF, while for low permeability drugs there will be only small gradients between brain ECF and CSF (Aird, 1984; Blasberg *et al.*, 1975; de Lange *et al.*, 1994; Patlak and Fenstermacher, 1975). Differences in spatial distribution due to differences in BBB transport have also been shown following local administration of drugs in the brain (de Lange *et al.*, 1995). Furthermore, processes like brain metabolism and effective diffusion through brain tissue will respectively increase and decrease the concentration gradient between CSF and brain ECF following direct administration into the CSF.

Then, bulk flow or convection of brain ECF, potentially by perivascular channels of flow, into the direction of the CSF is another means by which drugs can be redistributed within the brain. Finally, CSF does not present one compartment. Clear differences in pharmacokinetics of drugs may exist at the CSF from lumbar and ventricle sites (the ones most often used for obtaining CSF), due to diffusion but also CSF dynamics (Baker *et al.*, 1996; Balis *et al.*, 2000; Blaney *et al.*, 1995; Freund *et al.*, 2001; Kawakami *et al.*, 1994; Marsala *et al.*, 1995; Morikawa *et al.*, 1998).

In analogy to the effect of plasma component binding, also binding to brain constituents may have a huge impact on within brain distribution (Goldbaum and Smith, 1954; Kalvass *et al.*, 2007; Kurz and Fichtl, 1983). Altogether, the

relationships of the different clearances of a drug between blood and CNS, and within the compartments of the CNS will ultimately determine the concentrations of a drug within a specific part of the CNS.

ECF-ICF exchange

After passage of the BBB, a drug enters the extracellular space of the brain and may thereafter distribute into brain cells. In general, this intracellular distribution is quantitatively more profound for the more lipophilic drugs. Extracellular concentrations will depend on the relation between BBB- and extra-intracellular clearances for the unbound drug and the fraction of the drug bound within the brain cells.

Drug metabolism

Drug metabolism may occur at the level of extracellular and intracellular sites of the brain (Kerr *et al.*, 1984), but also at the the BBB and BCSFB (Ghersi-Egea *et al.*, 1988). Several enzymes that are involved in hepatic drug metabolism have been found in the small microvessels of the brain and the choroid plexus. Enzymes like CYP haemoproteins, several CYP-dependent monooxygenases, NADPH-cytochrome P450 reductase, epoxide hydrolase, and also conjugating enzymes such as UDP-glucuronosyltransferase and α-class glutathion S-transferase have been detected in blood vessels of the brain or closely surrounding cell types obtained from both rat and human brain tissue (Ghersi-Egea *et al.*, 1988; Ghersi-Egea *et al.*, 1994; Ghersi-Egea and Strazielle, 2001; Johnson *et al.*, 1993). For the BCSFB very high activities (similar to those in the liver) have been found for UDP-glucuronosyltransferase and epoxide hydrolase, while also several CYP isoenzymes are relatively highly expressed in the choroid plexus. Then for both barriers, α and μ classes of gluthathion S-transferase and glutathion peroxidase in relatively high values have been found (Ghersi-Egea *et al.*, 1994; Johnson *et al.*, 1993). All these enzymes may serve as "enzymatic barriers" to drug influx into the brain, and even may be inducible (Volk *et al.*, 1991). In how far drug metabolism at this level will have an impact on drug concentrations in the brain compartments remains to be elucidated (Kurata *et al.*, 1995).

Target site distribution

Most drugs exert their effects not within the plasma compartment, but within the target containing tissue(s). The process of drug distribution to the active site is reflected by the need of the "link-bridge" in many PK-PD relationships (Lin, 2006).

The pharmacological effect profile is determined by the concentration of drug at the target site or, more precisely, by the time course of target site drug concentration , (Danhof *et al.*, 2007; Eichler and Muller, 1998). For CNS compounds, an important question is whether the intra- or extracellular brain concentrations reflects the target concentration at best. For drugs such as corticosteroids (Falkenstein *et al.*, 2000), anticancer drugs and anti-HIV drugs (Fletcher, 1999; Peter and Gambertoglio, 1998), this is probably the intracellular concentration. However, most CNS active drugs have their target at extracellular recognition sites (membrane receptors), and for those drugs brain ECF concentrations may provide the most relevant information. Both BBB transport kinetics and intracellular distribution determine the concentration and time to equilibrium between concentrations in plasma and brain ECF (Liu *et al.*, 2005). In general, active transport out of the brain decreases and brain tissue binding increases the time to equilibrium in brain ECF. Apart from their role in BBB transport, active transporters may also play a role in ECF-ICF exchange of drugs because also at the brain parenchymal cells a number of transporters (P-gp and MRP) have been localised (Groenendaal, 2007; Lee *et al.*, 2001; Scism *et al.*, 2000).
Target site distribution may represent a rate-limiting step in the onset to the biological effect. This is reflected in a delay of the pharmacological effect relative to the drug concentration in plasma, which is often referred to as hysteresis (Danhof *et al.*, 2007). Intracerebral microdialysis is a useful tool in obtaining information on the kinetics of target site distribution of CNS compounds (de Lange *et al.*, 1997; Hammarlund-Udenaes *et al.*, 1997; Hammarlund-Udenaes, 2000; Xie *et al.*, 1999), which has been successfully applied to the characterisation of target site distribution kinetics especially in a number of investigations of the PK-PD correlation of morphine (Bouw *et al.*, 2001a; Groenendaal *et al.*, 2007a; Groenendaal *et al.*, 2008). Such knowledge is important for the progress in the development of novel mechanism-based target site distribution modeling concepts (Danhof *et al.*, 2007).

Target interaction

At the target-site in the brain, the relationship between the target site concentration of a drug and the response profile depends on several factors related to the drug and the biological system. Differences in such concentration-response relations are related to differences in receptor interaction kinetics (target affinity and intrinsic efficacy) but also on the biological system (i.e., the receptor density and the transducer function, relating receptor activation to

pharmacological response) (Danhof *et al.*, 2007; Kenakin, 1999). Therefore, the incorporation of receptor theory in PK-PD modelling for the prediction of *in vivo* drug concentration-response relationships is important as it enables a separation between drug-specific and biological system specific parameters (Van der Graaf and Danhof, 1997). Much of the conceptual framework regarding how to study receptor function evolved from the pharmacological investigation of drug action. A.V. Hill was the first to understand the relationship between drug concentration and response when he derived the Langmuir binding equation in the course of his studies on nicotine and curare (Hill, 1909). The occupancy theory evolved from then onwards and was refined to describe the effects of partial agonists (Ariens, 1954; Stephenson, 1956) and receptor reserve. The development of the occupancy theory has allowed the formulation of a series of mathematical models that describe the interaction of agonists and antagonists with their receptors, in terms of affinity and efficacy (Dougall, 2001). In the classical occupancy receptor theory, efficacy is a dimensionless proportionality constant denoting the ability of agonists to produce a pharmacological response. In theoretical terms, it is difficult to separate affinity and efficacy estimates of agonists for receptors (Kenakin, 1999). The operational model of agonism which describes the relationship between drug concentration, receptor interaction and response, was proposed by Black and Leff (Black and Leff, 1983). Concepts from receptor theory have been applied in the development of a PK-PD modeling strategy that constitutes a scientific basis for the prediction of *in vivo* drug concentration-effect relationships, for example in the PK-PD analysis of neuroactive steroids (Visser *et al.*, 2002), benzodiazepines (Tuk *et al.*, 1999; Tuk *et al.*, 2002; Visser *et al.*, 2003b), adenosine A1 receptor agonists (Van der Graaf *et al.*, 1997), 5-HT1A receptor agonists (Zuideveld *et al.*, 2004), opioids (Groenendaal, 2007; Yassen *et al.*, 2005) and β-blockers (van Steeg *et al.*, 2008). The incorporation of receptor theory in PK-PD modeling has recently been reviewed (Ploeger *et al.*, 2009)

5. Sources of Variation in Mechanisms Contributing to the Response Profile

The mechanisms on the causal path between dose and response of CNS drugs, as described above, can be viewed as being placed in series. Each mechanism may influence the CNS drug response. Here we will discuss sources of variation in individual mechanisms, showing that changed conditions may change the mechanism(s) which detremine the relationship between dose and response. For interpretation, comparison and extrapolation of research data, one should be able to identify the sources of variation in the individual mechanisms.

In most neuropharmacological studies the effect of drugs in the CNS are still related to the dose (Girgin *et al.*, 2008; Homma *et al.*, 2008; Kinon *et al.*, 2008; LeWitt *et al.*, 2008; Mischoulon *et al.*, 2008; Stern *et al.*, 2004; Walsh *et al.*, 2008).

However, a unique dose-effect relationship it is not generally applicable. This is because the above-mentioned mechanisms do not contribute equally in different conditions. The individual mechanisms may differ quantitatively due to differences in genetics, species, gender, age, environmental and pathological conditions etc. Thus, knowledge of the variability in these mechanisms under different circumstances, as well as their interplay is important in making comparisons between different conditions or for making predictions of drug response under certain conditions (Figure 4).

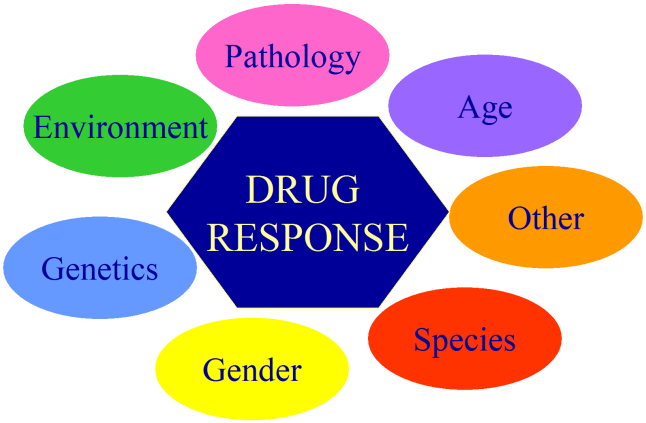

Figure 4: *Sources of variation in mechanisms between dose and response.*

Species

In drug development many pharmacological and toxicological studies are performed in small laboratory animals such as mice, rats, rabbits, dogs and monkeys. Species differences and therefore interspecies scaling is an important issue for the prediction of pharmacokinetic parameters from these animals to humans. The onset, intensity and duration of drug response depend on the absorption, distribution, metabolism and elimination that are inherent to the biological system (Hurst *et al.*, 2007). In addition to metabolic differences, the anatomical, physiological, and biochemical differences in the gastrointestinal tract (i.e. transit time, pH, microbial content, membrane transport) of the human and common laboratory animals can cause significant variation in drug absorption from the oral route (Kararli, 1995).

Serum albumins from mammalian species differ in amino acid sequence and protein structure. Consistent with this, binding-site affinity and selectivity vary from species to species as was observed for warfarin in rat, bovine, and human albumin. Because of these differences, albumins from separate species should not be considered interchangeable (Mandula *et al.*, 2006).

In general, small animals tend to eliminate drugs more rapidly than human beings when compared on a weight-normalised basis. There are relatively small differences in the primary amino acid sequences of the CYP enzymes across species, although these might have an impact on substrate specificity and catalytic activity and consequently on drug metabolism across species. Particularly CYP1A, CYP2C, CYP2D and CYP3A appear to show interspecies difference (Martignoni *et al.*, 2006).

With regard to the brain, species differences have been shown in brain microvessel MAO, with rat microvessels having one of the highest MAO activity among all tissues, whereas MAO activities in brain microvessels from humans, mice, and guinea pigs were very low (Kalaria and Harik, 1987). Species differences were also found in the brain and brain-to-plasma concentrations of of three P-gp substrates using PET technology showing higher brain distribution in humans, monkeys, and minipigs than in rats and guinea pigs. The species differences were still present after P-gp inhibition, specifically in rats. Differences in plasma protein binding or metabolism did not explain the species-related differences (Syvanen *et al.*, 2009).

Other physiological parameters, such as body temperature (36°C - 38°C), and haematocrit (40% - 45%) are relatively conserved among animals and are independent of animal size (Davies and Morris, 1993).

Genetics

Genetic variability in drug response occurs as a result of molecular alterations at the level of drug-metabolising enzymes (pharmacokinetics), drug transport molecules that mediate drug uptake into and efflux from intra- and or extra-cellular sites (pharmacokinetics) and drug targets/receptors (pharmacodynamics) (El Desoky *et al.*, 2006; Ensom *et al.*, 2001). The pharmacokinetic and/or pharmacodynamic consequences of gene polymorphisms may include increased risk of toxicity or a change in response (Shah, 2005).

Metabolising enzymes

The most widely known example in genetic polymorphisms of metabolising enzymes are in the cytochrome P450 enzymes (CYPs). Specifically CYP2D6, CYP2C19 and CYP2C9 are of interest as they metabolise a substantial portion of all the drugs used (Ensom *et al.*, 2001) with CYP2D6 being involved in the metabolism of more than 30% of CNS drugs such as cholinesterase inhibitors, antidepressants, neuroleptics, opioids and many others (Cacabelos, 2008a). CYP2D6 is also the main metabolising enzyme for many antiarrhythmic drugs. Cardiac patients who were phenotyped as being poor metabolisers for CYP2D6, have been found at high risk of severe side effects or drug toxicity when using propafenone or mexiletine (El Desoky *et al.*, 2006). Ultrarapid and extensive metabolisers for CYP2D6 are at risk of side effects caused by the active metabolite of the antiarrhythmic drug encainide. Phenytoin, used in the treatment of epilepsy, undergoes significant metabolism by CYP2C9 and CYP2C19 and is able to induce intoxication in patients with polymorphisms in these enzymes (Anderson, 2008b). Although the CYP enzymes are the most widely studied and best characterised metabolic enzymes, polymorphic variants can also occur in various dehydrogenases, esterases, NADPH-quinone oxidoreductase UDP-glucuronyltransferases (UGT), various methyltransferases and sulfotransferases (Cacabelos, 2008b). Differences in catechol-O-methyltransferase (COMT) activity (one of the metabolising enzymes for L-DOPA) and genotype may determine individual variations in the therapeutic response to L-DOPA or Parkinson's disease susceptibility (Bialecka *et al.*, 2008b).

Transporters

P-gp is a MDR-1 gene product and several polymorphisms have been reported, some of which might affect P-gp expression and function (Hoffmeyer *et al.*, 2000) and could lead to elevated drug levels in many tissues (Schinkel *et al.*, 1994). P-gp

polymorphisms have also been associated with increased risk for Parkinson's disease (Furuno *et al.*, 2002; Lee *et al.*, 2004). Bartels *et al* claimed a decreased P-gp function in patients with Parkinson's disease (Bartels *et al.,* 2008). Differences have been observed in pharmacokinetic parameters of digoxin between different genotypes of the MDR-1 gene (Johne *et al.*, 2002; Kurata *et al.*, 2002). Higher concentrations of rhodamine-123, dexamethasone, digoxin, cyclosporin A, ondansetron, loperamide and morphine were observed in brain tissue of mdr1a(-/-) mice compared to wild-type mdr1a(+/+) mice (de Lange *et al.*, 1998; Meijer *et al.*, 1998; Schinkel *et al.*, 1995; Schinkel *et al.*, 1996) incidating the consequence of the absence of functional P-gp.

Targets

Variant alleles are known to occur not only at the genes expressing target enzymes, channels and receptors but also at the genes responsible for intracellular signal transduction (Shah, 2005). There is evidence to indicate that the β_2-adrenergic receptor is polymorphically expressed and that this contributes to the interindividual variability in response of patients with asthma to certain drugs. Similar evidence has been found in the promotor region of the serotonin transporter with a consequence in the response to fluvoxamine (Shah, 2005). In a study involving twins, about 30% to 35% of variance in heart rate acceleration as induced by a 30-minute i.v. infusion of nicotine and cotinine was due to genetic sources (Swan *et al.*, 2007). The presence of APOE-4 allele in patients suffering from Alzheimer's Disease (AD), differentially affects the quality and extent of drug responsiveness when these patients were treated with cholinergic enhancers such as galantamine or with non-cholinergic drugs (Cacabelos, 2008a).

Gender

In general, females experience more adverse drug reactions (ADR) than males (Zopf *et al.*, 2008). Hormonal fluctuations in the menstrual cycle or pregnancy are considered to be the main reason for gender differences in the pharmacokinetics and pharmacodynamics of drugs. Evidence for physiological variation within the menstrual cycle is limited. Variations have been observed in the renal, gastrointestinal and cardiovascular systems as well as in plasma lipids and haematological and immune function (Kashuba and Nafziger, 1998). Also, variability during the menstrual cycle has been observed for α_1-acid glycoprotein. The influence of the menstrual cycle on the pharmacokinetics of drugs has been

shown for e.g. paracetamol (acetaminophen) and ranitidine (Flores *et al.*, 2003; Gugilla *et al.*, 2002) but was not seen for triazolam or caffeine (Kamimori *et al.*, 1999; Kamimori *et al.*, 2000).

Non-hormonal or reproductive-related differences between male and female subjects can be either physiological (body weight, body fat, glomerular filtration, organ size, gastric motility) or molecular (drug transporters such as the dopamine transporter (Dluzen and McDermott, 2008) or drug-metabolising enzymes).

In general, females weigh less than males, so they often receive higher doses which results in higher drug exposure. In the example of L-DOPA, used in the treatment of Parkinson's disease, this difference in bodyweight might be the reason for the difference in the proportion of men and women experiencing dyskinesias during the course of the disease (Martinelli *et al.*, 2003; Zappia *et al.*, 2002). Estrogen has been shown to inhibit COMT (Shulman, 2007) which would explain the higher exposure to L-DOPA in women. Furthermore, females have a higher percent body fat than males which can affect the volume of distribution of certain drugs. Differences in the activity of hepatic enzymes (specifically CYP3A4 (Greenblatt and von Moltke, 2008)), drug transporters and renal excretion between males and females could result in differences in elimination of a drug (Anderson, 2008a). P-gp has been mentioned as a drug transporter whose activity might be different in males and females but literature is contradicting on this point (Schuetz *et al.*, 1995; Wolbold *et al.*, 2003). Differences in response to drugs between men and women have been seen for various drugs, such as opiates (Dahan *et al.*, 2008), anti-HIV drugs (Floridia *et al.*, 2008), paroxetine (Gex-Fabry *et al.*, 2008), midazolam (Sun *et al.*, 2008) and citalopram (Young *et al.*, 2008).

Age

Roughly three age-groups can be distinguished among patients, namely paediatric, adult and geriatric patients. Both paediatric patients (0-18 years of age) and geriatric patients (>65 years) are considered special populations in drug discovery and development. Most drugs are first developed for the adult patient population (19-65 years) with studies subsequently performed in elderly and children. This review will not emphasize on drug disposition in the paediatric population, as this is irrelevant for Parkinson's disease and reviewed elsewhere (Kearns *et al.*, 2003).

In elderly patients drug response is not only dependent on physiological changes which are associated with age but the effects of disease and of comedications should also be taken into consideration. In general, drug absorption from the gut

is usually not diminished in elderly. However, compounds that cross the intestinal epithelium by carrier-mediated transport mechanisms might be absorbed at a lower rate in the elderly as well as for compounds which have been administered via the dermal, subcutaneous or intramuscular route, due to reduced tissue blood perfusion (Turnheim, 2003). Skeletal muscle mass and total body water content declines with age but total body fat increases with age which might have implications for the volume of distribution of a drug (for hydrophilic drugs it decreases and for lipophilic drugs it increases) and consequently on the half-life of a drug (Turnheim, 1998).

Despite significant research efforts, the effect of age on hepatic drug metabolism continues to be a controversial issue. Aging was found to be associated with a reduction in liver weight of about 25% - 35% (Le Couteur and McLean, 1998). Liver function, as measured via routine tests, however, does not change significantly with advancing age, although serum albumin can decrease slightly. Liver blood flow declines by about 40% with aging. Bile flow and bile salt formation are reduced by about 50% (Fu and Nair, 1998; Herrlinger and Klotz, 2001; Le Couteur and McLean, 1998). At all ages there is a wide genetic variability in the rates of drug clearance, specifically within the CYPs as described previously in this review. Interindividual variability in drug clearance accounts for the problems in detecting any changes due to aging (Zeeh and Platt, 2002)

The most pronounced pharmacokinetic change in elderly is the reduction in renal elimination of drugs due to reduced renal function. The glomerular filtration rate declines by 25% - 50% between the ages of 20 and 90. Because of these changes, an age-dependent decline of total clearance is to be expected for all drugs that are predominantly eliminated by the kidneys (Herrlinger and Klotz, 2001). Other physiological changes with age include altered structure of proteins (Gafni, 1997), altered pulmonary function, reduced cardiac output, decrease in serum levels of various hormones and decline in the immune system (Turnheim, 2003). Moreover, phase-contrast magnetic resonance imaging has shown a significant decrease of total cerebral blood flow in elderly individuals with a linear correlation with age (Stoquart-ElSankari *et al.*, 2007). Many studies have demonstrated substantial and important age-related changes in neurochemistry and neurobiology. Data on the decreases and alterations in the dopaminergic and serotonin neurotransmitter pathways have been relatively consistent in both animal and human studies (Adolfsson *et al.*, 1979; Bucht *et al.*, 1981; Wenk *et al.*, 1989). The numbers of dopamine D1 and D2 receptors, as well as serotonin 5HT1A and 5HT2A receptors are reduced in several brain regions with aging. Studies in other receptors show

that other neuronal systems and receptors are affected by age-related alterations as well (Schwartz and Abernethy, 2009). The changes in these neurotransmitter and receptor systems may account for, in addition to changes in drug reaction and metabolism, alterations in behaviour, cognitive abilities, and mood in the elderly. Other CNS signaling systems-the GABAergic (i.e., producing γ-aminobutyric acid) and enkephalin-endorphin system have not been well studied in older individuals; however, clinical studies of drugs acting on these systems have noted age-related changes in their pharmacodynamics (Schwartz and Abernethy, 2009).

Environmental factors

Cigarette smoking and other environmental factors have been shown to influence the metabolism of some drugs (Vestal and Wood, 1980; Wood *et al.*, 1979). Cigarette smoking reduces the therapeutic response to certain drugs such as theophylline through the induction of hepatic cytochrome P450 isoenzymes (Braganza *et al.*, 2008). Polycyclic aromatic hydrocarbons (PAHs) are some of the major lung carcinogens found in tobacco smoke. PAHs are potent inducers of the hepatic CYP1A1, CYP1A2, and, possibly, CYP2E1. Other compounds such as acetone, pyridine, heavy metals, benzene, carbon monoxide, and nicotine may also interact with hepatic enzymes but their effects appear to be less significant. The primary pharmacokinetic interactions with smoking occur with drugs that are CYP1A2 substrates, such as caffeine, clozapine, fluvoxamine, olanzapine, tacrine, and theophylline. The primary pharmacodynamics drug interactions with smoking are hormonal contraceptives and inhaled corticosteroids (Kroon, 2007). Cigarette smoking can affect the pharmacokinetic and pharmacodynamics properties of many psychotropic drugs as summarised elsewhere (Desai *et al.*, 2001).

Pharmacokinetic interactions between food and orally administered drugs involve (1) before and during gastrointestinal absorption; (2) during distribution; (3) during metabolism; and (4) during elimination (Singh, 1999). Absorption and metabolism are the phases where food has most effect and this may have clinical implications for drugs such as in the treatment of cancer (Singh and Malhotra, 2004). Food can decrease the rate of L-DOPA absorption, but has no effect on the systemic exposure to L-DOPA and the degree of 3-O-methyldopa formation (Crevoisier *et al.*, 2003).

Pathological conditions

Disease is defined as "any deviation from or interruption of the normal structure

or function of any body part, organ, or system that is manifested by a characteristic set of symptoms and signs" (The Free Dictionary, 2009). Given this, alteration(s) at any place in the body as a result of a pathological condition might influence the pharmacokinetics and/or the pharmacodynamics of a drug. Some general examples are given below. The distict example of Parkinson's disease is discussed later in this review.

Obesity is a condition in which excess body fat has accumulated to such an extent that health may be negatively affected. It is commonly defined as a body mass index (BMI) of 30 kg/m^2 or higher. This distinguishes it from being overweight as defined by a BMI of 25 kg/m^2 or higher. Cardiac performance and adipose tissue blood flow may be altered in obesity. There is uncertainty about the binding of drugs to α_1-acid glycoprotein in obese patients. Some data suggest that the activities of hepatic CYPs are altered, but no clear overview of drug hepatic metabolism in obesity is currently available. Pharmacokinetic studies provide differing data on renal function in obese patients (Cheymol, 2000).

Alterations of drug transporters, as well as metabolic enzymes, in patients with chronic renal failure (CRF) can be responsible for reduced drug clearance. CRF not only alters the renal elimination of drugs. Pharmacokinetic studies conducted in patients with CRF demonstrate that the nonrenal clearance of multiple drugs is reduced. CRF affects the metabolism of drugs by inhibiting key enzymatic systems in the liver, intestine and kidney. The down-regulation of CYPs has been reported next to a decrease in gene expression. Liver phase II metabolic reactions are also reduced in CRF. Moreover, intestinal drug disposition is affected in CRF. Increased bioavailability of several drugs has been reported in CRF, reflecting decrease in either intestinal first-pass metabolism or extrusion of drugs (mediated by P-glycoprotein). Decreased activity of P-gp was observed in CRF rats with no significant change of protein content, suggesting that uremic toxins may suppress P-gp function. With the development of CRF, the renal secretion of organic ions mediated by OAT and OCT is decreased. Organic anionic uremic toxins may directly inhibit the renal excretion of various drugs and endogenous organic acids by competitively inhibiting OAT. In addition, the expression of OAT1 and OCT2 was reduced in chronic CRF rats (Pichette and Leblond, 2003; Sun *et al.*, 2006).

The capacity of the liver to metabolise drugs depends on hepatic blood flow and liver enzyme activity, both of which can be affected by liver disease. Liver disease can modify the kinetics of drugs biotransformed by the liver. Studies on the effects of liver disease on specific isoenzymes of CYP have shown that some isoforms are

more susceptible than others to liver disease. In addition, liver failure can influence the binding of a drug to plasma proteins which in turn could influence the processes of distribution and elimination. Portal-systemic shunting, which is common in advanced liver cirrhosis, may substantially decrease the presystemic elimination (i.e., first-pass effect) of high extraction drugs following their oral administration, thus leading to a significant increase in the extent of absorption. Glucuronidation is often considered to be affected to a lesser extent than CYP-mediated reactions in mild to moderate cirrhosis but can also be substantially impaired in patients with advanced cirrhosis (Rodighiero, 1999; Verbeeck, 2008).

Other

Circadian rhythms can influence the pharmacokinetics of a drug which has been shown for over 100 drugs, among them is L-DOPA, for which a higher plasma clearance and lower area under concentration curve was observed in rats after the administration in the late evening (Andre *et al.*, 1996). Absorption may be influenced and most lipophilic drugs seem to be absorbed faster when the drug is taken in the morning compared with the evening; for water-soluble compounds, no circadian variation in the absorption of drugs has been found. Concerning drug distribution, the higher the blood flow fraction an organ receives, the higher the rate constant for transferring drugs out of the capillaries. This drug pharmacokinetic phase may be influenced by circadian variations in the protein binding of acidic and basic drugs. Drug metabolism may be influenced by daily modifications of blood flow. For drugs with a high extraction ratio, metabolism depends on hepatic blood flow, while that of drugs with a low extraction ratio depends on liver enzyme activity. Hepatic blood flow has been shown to be greatest at 8 am and metabolism seems to be reduced during the night. Finally, concerning drug elimination, the clearance of 'flow-limited' drugs that present a high extraction rate is affected by the blood flow delivered to the organ, independent of the cardiac output fraction supplied (Baraldo, 2008). As the cardiovascular system is the means of transport, blood flow fraction destined to each organ determines the relative mass of solute in plasma, which is constantly in contact with the tissue. Hence, not only the rate but also the extent of drug transfer would be increased when tissues are irrigated by a higher fraction of cardiac output. Aging and circadian rhythms present similar cardiac output distribution patterns when moving from young to aged adult and from nocturnal to diurnal hours. These two changes lead to an increased blood flow delivery to the renal region in the elderly and in the morning, but with a decreased cardiac

output in aged individuals and an increased one during the day. This scenario allows us to forecast substance concentrations outside the blood vessels, which are responsible for the extent of drug elimination and the intensity of drug effect (Fagiolino *et al.*, 2006).

Body position may influence physiological characteristics, such as perfusion, gastrointestinal function and plasma volume. These characteristics may interact with key factors determining the pharmacokinetics of drugs. Postures which favor rapid gastric emptying accelerate the absorption of orally administered drugs. Consequently, these postures favour a shorter time to reach peak plasma drug concentration and a higher C_{max} and-in the case of transient saturation of first-pass metabolism-total exposure (area under the concentration-time curve, AUC) in comparison to recumbent left and supine. For highly protein-bound drugs (e.g. phenytoin, imipramine), the total plasma concentration has been found to be approximately 10% higher in standing than lying subjects due to changes in plasma volume (Queckenberg and Fuhr, 2008).

Research has shown ethnic differences in the clinical presentation, treatment, clinical response and outcome of mental illnesses. A number of ethnically specific variations have been found in the genetic and non-genetic mechanisms affecting pharmacokinetics and dynamics of psychotropic drugs, which might underlie the differences in drug use and response across ethnicities. Although some of these ethnic differences could be partially explained by genetic factors, a number of ethnically based variables like culture, diet and societal attitudes could potentially have a significant, but as yet unquantified influence as well (Chaudhry *et al.*, 2008). The pharmacokinetic factors which can be expected to potentially exhibit racial differences are (1) bioavailability for drugs which undergo gut or hepatic first-pass metabolism, (2) protein binding, (3) volume of distribution, (4) hepatic metabolism, and (5) renal tubular secretion. Absorption (unless active), filtration at the glomerulus, and passive tubular reabsorption would not be expected to exhibit racial differences (Johnson, 1997).

6. The BBB in Neurodegeneration: Implications for PK-PD Relationships of Antiparkinson Drugs

The BBB is a key role player in the relationship between plasma and brain pharmacokinetics as the rate of penetration into the brain tissue is limited by the diffusion of the drug across the BBB. Drug transport to the brain is dependent on physico-chemical properties of the drug suchs as lipophilicity, ionistation and pH in relation to membrane properties. The BBB behaves differently from most other membranes in the body due to the presence of tight junctions and active influx and efflux transporters in the membrane resulting in an unequal (unbound) drug concentration on both sides of the BBB at steady state. Other factors may also contribute to this phenomenon, such as metabolism within the brain, or drug transport to the CSF via interstitial fluid (ISF) bulk flow (Hammarlund-Udenaes *et al*., 2008). In fact, for many drugs (both lipophilic and hydrophilic) unbound brain concentrations are much lower than the corresponding blood concentrations (Hammarlund-Udenaes *et al.*, 1997). Hammarlund-Udenaes and collegues (Hammarlund-Udenaes *et al.*, 1997) have performed simulations using a model with one body compartment and one brain compartment to describe unbound brain concentration-time profiles in relation to unbound blood profiles to

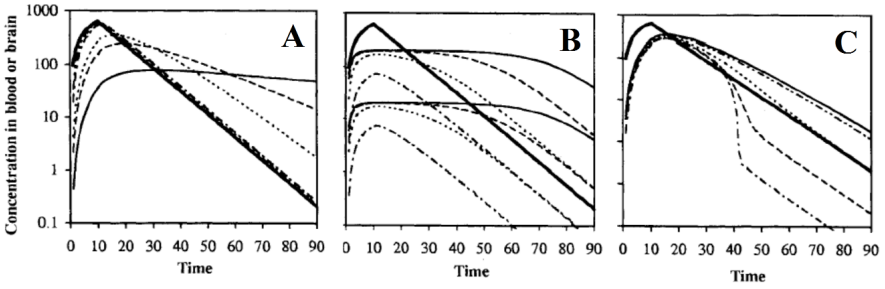

Figure 5: Simulations of brain concentrations. In all figures, the thick line depicts the blood concentration (*taken with permission from Hammarlund-Udenaes et al 1997;Pharm. Res. 14:128-134)*

A: Passive diffusion into and out of the brain.
Cl_{in} = Cl_{out} and decreases from 1 (-··-··-), 0.5 (-·-·-), 0.1 (·····), 0.05 (---) and 0.01 (—)
B: Active, saturable transport into the brain and passive transport out of the brain.
Cl_{out} = 0.5; $T_{m,in}/K_{m,in}$ varies from 100/1 & 10/1 (—), 100/10 & 10/10 (---), 100/100 & 10/100 (·····) and 100/1000 &10/1000 (-·-·-)
C: Active, saturable transport out of the brain and passive transport into the brain.
Cl_{in} = Cl_{out} = 0.1; $T_{m,out}/K_{m,out}$ varies from the top 10/1000 (-·-·-), 10/100 (·····), 10/10 (---) and 10/1 (-·-·-)

investigate the effect of changes in passive and active transport into and out of the brain. Figure 5A shows the simulation of passive diffusion across the BBB (Cl_{in}=Cl_{out}). Decreasing Cl_{in} and Cl_{out}, which simulates increasing hydrophilicity of a drug (or a decrease in BBB permeability), results in a higher t_{max} and longer half-life in the brain. Active, saturable transport into the brain and passive transport out of the brain is depicted in Figure 5B. Cl_{out} is fixed in this simulation and $T_{m,in}/K_{m,in}$ varies (T_m is the maximal transport rate and $K_{m,in}$ is the blood concentration at half-maximal transport). A higher T_m results immediately in higher brain concentrations, whereas a decrease in $K_{m,out}$ compared to blood concentration results in brain concentrations to remain at a constant level for a longer period of time. Active, saturable transport out of the brain and passive transport into the brain is depicted in Figure 5C. Here, Cl_{in} = Cl_{out} and is fixed and $K_{m,out}$ in $T_{m,out}/K_{m,out}$ varies ($K_{m,out}$ is the brain concentration at half-maximal transport). With decreasing $K_{m,out}$, the AUC_{brain}/AUC_{blood} is smaller. These simulations also give insight into altered brain concentrations of drugs due to possible disease-induced changes in BBB transport characteristics.

As already described above, BBB transport is related to BBB functionality and occurs by passive diffusion as well as by active transport. BBB functionality is dynamically controlled by blood components and the surrounding brain cells by direct contact or indirectly by their extracellular products. Thus, BBB functionality may vary among different physiological, pathological, and chronic drug treatment conditions. As BBB transport of a particular CNS drug into and out of the brain is the sum of all actual BBB transport mechanisms applicable to that particular drug, any changes in BBB transport mechanisms may therefore affect actual BBB transport as simulated in Figure 5 and therewith the effects of the drug. This may also apply for neurodegenerative diseases like Parkinson's disease. PK-PD relationships of symptomatic drugs such as L-DOPA in patients with different stages of Parkinson's disease have been described previously (Chan *et al.*, 2005; Harder and Baas, 1998) and indicate that Parkinson's disease progression is able to influence the PK of anti-Parkinsonian drugs in plasma. It has been known for many years that the BBB is less effective during aging. The transport systems involved in the transport of e.g. choline, glucose, lactate or peptides across the BBB have shown to be affected by age (Banks and Kastin, 1985; Mooradian, 1988; Mooradian, 1994). Common age-related changes of the BBB are reduced thickness of endothelial cells, vacuolar inclusions in pericytes and decreased release of amino acids in the cerebral parenchyma (Barcia *et al.*, 2004). It has also long been proposed that neurodegeneration is associated with changes

in the functional and biochemical integrity of the BBB (Mooradian, 1988; Pardridge, 1988a). Evidence has been found in the break-down of the BBB in patients with AD (Algotsson and Winblad, 2007; Bowman *et al.*, 2007; Desai *et al.*, 2007), more specifically in white matter (Tomimoto *et al.*, 1996) and in biopsy tissue from AD patients (Claudio, 1996). Moreover, age-related changes in the morphology of capillaries located in the white matter of dogs are thought to be associated with BBB dysfunction (Morita *et al.*, 2005) as well as the presence of micro-angiogenesis in the Parkinson's diseased brain (Barcia *et al.*, 2004). An increase of vascular endothelial growth factor and increase of blood vessels in the SNc of Parkinson's disease patients have been observed (Barcia *et al.*, 2005) and pathological changes in capillary microanatomy in patients with Parkinson's disease and AD have been shown (Farkas *et al.*, 2000). These studies all suggest that neovasculation is observed in the (Parkinson's) diseased brain, which could result in the disruption of the BBB. Monahan and colleagues propose that Parkinson's disease is, in part, an autoimmune disease as a disrupted BBB could result in the entry of immune cells leading to a progressive degenerative process (Monahan *et al.*, 2008).

Several studies have shown that oxidative stress in the SNc is associated with inflammatory processes such as increase of microglia activation (Hirsch *et al.*, 2005; McGeer and McGeer, 2004a) and increase levels of pro-inflammatory cytokines like tumor necrosis factor (TNF)-alpha, interleukin (IL)-1beta, and IL-6 (Teismann *et al.*, 2003). In meningitis and sepsis, inflammation can disrupt the function of the BBB, and this has also been shown in trauma, stroke, multiple sclerosis and epilepsy (Huber *et al.*, 2001). The activated microglia can stimulate the release of TNF-alpha, and this pro-inflammatory cytokine can disrupt the barrier function *in vitro* and *in vivo* (Lynch *et al.*, 2004; Mark and Miller, 1999; Tsao *et al.*, 2001). Carvey *et al* have suggested that activation of microglia released TNF-alpha leading to the breakdown of BBB (Carvey *et al.*, 2005). They have demonstrated the leakage of fluorescein isothiocynate (FITC)-labeled albumin and horseradish peroxidase in the SNc and striatum of 6-OHDA-lesioned rats and associated it with dopamine neuron loss, activated microglia, presence of neovascularisation and the increase entry of small molecules into the brain. Also, the areas of leakage in the BBB were associated with increased P-gp expression. At the same time, a reduced P-gp function in patients with Parkinson's disease was claimed using PET to measure brain uptake of [^{11}C]-verapamil (Bartels *et al.*, 2008; Kortekaas *et al.*, 2005). However, the elevated uptake of [^{11}C]-verapamil could also be related to an increase in paracellular transport of the compound

instead of a reduced P-gp function as Carvey *et al* demonstrated in the same study that domperidone, a dopamine antagonist which is normally not transported into the brain, was able to inhibit dopamine-mediated behaviour in the 6-OHDA lesioned rats (Carvey *et al.*, 2005). Futhermore, 6-OHDA rat models with L-DOPA induced dyskinesia have demonstrated that dyskinesias in animals were associated with increased entry of L-DOPA into the striatum (Carta *et al.*, 2006; Westin *et al.*, 2006) which was also seen using PET imaging in patients with peak-dose dyskinesias at 1 hour after L-DOPA administration (Fuente-Fernandez *et al.*, 2004). The dyskinetic 6-OHDA rats also exhibited a significant increase in total blood vessel length and a visible extravasation of serum albumin in the SNc (Westin *et al.*, 2006), indicating a role for the BBB in the altered transport of L-DOPA to the brain.

7. Summary and Concluding Remarks

Parkinson's disease is a progressive neurodegerative disease that lacks good treatment especially at later stages. Apart from plasma pharmacokinetics, mechanisms that govern CNS drug distribution and response include the rate and extent of BBB transport and the kinetics of distribution within the brain including the brain target distribution. For the development of new drugs as well as for the optimisation of therapy with the current drugs, the variability of these individual mechanisms and contribution in terms of rate and extent should be investigated. As the BBB is a key player in the relationship between plasma and brain pharmacokinetics, the influences of disease states on BBB functionality in the various stages of the disease is important in order to judge on drug effects. This warrants the application of a systems pharmacology approach in investigations on variability in drug response in Parkinson's disease.

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Chapter 3

Animal Models as a Tool in Systems Pharmacology Research on Parkinson's Disease

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Abstract

Parkinson's disease is a progressive neurodegenerative disease, which is composed of many components, each caused by interplay of a number of genetic and nongenetic causes. This warrants a systems pharmacology approach to the development of novel drug treatments of Parkinson's disease. Animal models of disease are an essential asset in this research.

There is no single animal model which reflects all aspects of Parkinson's disease. The available animal models however may reflect specific aspects, making each animal model suitable for specific Parkinson's disease research questions. A combination of models may provide pertinent information on Parkinson's disease characteristics and treatment effects in the system as a whole.

Specifically, an animal model for Parkinson's disease displaying the slow progressive nature of the disease constitutes a basis to obtain the essential biological system-specific information to determine the neuroprotective properties of new drugs, preclinically.

In this review we have summarised the currently available animal models of Parkinson's disease, with their main characterstics, followed by a discussion on the *in vivo* monitoring technique microdialysis, to obtain information on the target site distribution. Here, the emphasis is on blood-brain barrier (BBB) functionality in Parkinson's disease and the added value of intracerebral microdialysis to assess disease related changes in BBB functionality affecting drug transport into and out of the brain, and the within brain distribution to the target site. Furthermore, to be able to determine the effect of drugs on the Parkinsonian behaviour in animal models, we have summarised the most commonly used behavioural tests used. Finally, the state of the art of mechanism PK-PD modelling is introduced as novel systems pharmacology approach to characterise drug effects *in vivo*.

1. Introduction

Parkinson's disease is a neurological disorder characterised by resting tremors, rigidity, bradykinesia and postural instability. These symptoms are a result of the loss of dopamine-producing neurons in the substantia nigra pars compacta (SNc) and a depletion of dopamine in the striatum. Clinical manifestations do not occur until there is a loss of approximately 50% - 80% of the dopaminergic neurons within the SNc and a loss of about 70% of the striatal dopamine (Bohlen Und, 2005; Dauer and Przedborski, 2003). A further histopathological hallmark of the disease is the presence of intracytoplasmic inclusions called Lewy bodies (LBs) in

the remaining dopaminergic neurons of the SNc. The major compounds in these eosinophilic LBs are aggregated forms of the normally presynaptically located protein α-synuclein (Bohlen Und, 2005; Dauer and Przedborski, 2003).
Today's treatment of the symptoms of Parkinson's disease consists predominantly of replacing the lost brain dopamine by means of the dopamine precursor L-DOPA (Mercuri and Bernardi, 2005), in combination with a peripheral decarboxylase inhibitor. To overcome motor complications associated with long-term treatment with L-DOPA, which occur in a later stage of the disease, L-DOPA is often administered in combination with either a dopamine agonist, a catechol-O-methyltransferase (COMT) inhibitor, or a monoamine-oxidase-B (MAO-B) inhibitor. However, the further the progression of Parkinson's disease the less effective symptomatic drug treatment becomes and the more complications occur. It is clear that there is a need for drugs that can reduce or even halt disease progression in Parkinson's disease by using drugs that have neuroprotective or neurorestorative properties (Bonuccelli and Del Dotto, 2006; Chen and Le, 2006; Schapira, 2008). Some of the drugs already in use as treatment in Parkinson's disease (e.g. dopamine agonists), or currently under investigation (e.g. A2A antagonists), seem to offer some neuroprotective benefits (Bonuccelli and Del Dotto, 2006; Olanow, 2009). However, to date no drug has been proven to be neuroprotective in Parkinson's disease (Olanow, 2009) or has been approved for a neuroprotective indication (Rascol, 2009). A major limitation in this respect is the lack of an endpoint that accurately reflects the underlying disease state (Olanow, 2009) while also no 'optimal' clinical trial design is readily available for studying neurprotective properties of drugs in Parkinson's disease (Rascol, 2009; Stocchi and Olanow, 2003). For estimating the actual treatment effect a clinical trial should allow the time course of treatment effects to be distinguished from those due to natural disease progression (Ploeger and Holford, 2008). Other obstacles include the absence of precise knowledge on the etiology and pathogenesis of Parkinson's disease. Parkinson's disease, like other neurodegenerative diseases, consists of many disease components, each caused by an interplay of a number of different genetic and nongenetic causes (Gasser, 2009).

Investigation on the interplay between these disease components and drug treatment efficiency cannot be studied *in vitro* alone, nor in the human situation. This implies the need for *in vivo* animal studies. Indeed, animal models of Parkinson's disease (or neurodegenerative diseases in general) have their restrictions, as there will be no animal model reflecting all aspects of this disease.

As a result it has been stated that there is a lack of relevant animal models (Kieburtz and Olanow, 2007). However, various animal models of Parkinson's disease reflect particular aspects of the disease, making each animal model suitable for specific Parkinson's disease research questions, while a combination of models may provide important answers on Parkinson's disease features and treatment effects in the system as a whole.

Mechanism-based pharmacokinetic-pharmacodynamic (PK-PD) models are useful here in the sense that they aim to characterise, in strictly quantitative manner drug effects *in vivo* under physiological and pathological conditions. To this end mechanism-based PK-PD models contain specific expression to characterise processes on the causal path between drug administration and response (e.g. target site distribution, target binding and activation, transduction/homeostatic feedback and diseases processes/progression). In the meantime it has been demonstrated that mechanism-based PK-PD models have excellent properties for extrapolation and prediction in systems pharmacology research (Danhof *et al.*, 2007). Systems pharmacology aims at the development of an understanding of the interactions between pathophysiology and drug action. To develop such an understanding it is necessary to analyse interactions across and between various scales of organisation (Wist *et al.*, 2009). To date most PK-PD modeling research has focused on healthy systems. A major challenge is the characterisation of drug effects on disease processes and disease progression. A particularly intriguing challenge is the prediction of drug effects on disease progression in man from preclinical research. For this, the development of chronically instrumented animal models to determine the time course of drug concentration at the target site along with the effect on disease progression is very important. An animal model for Parkinson's disease displaying the slow progressive nature of the disease is essential and would provide the necessary biological system specific information to determine the neuroprotective properties of new drugs, preclinically. Relevant biomarkers will enable a detailed and mechanistic description of disease progression and serve as useful tools in these disease models. Within the context of mechanism-based PK-PD modeling, a biomarker is defined as a measure that characterises, in a strictly quantitative manner, a process, which is on the causal path between drug administration and effect (Danhof *et al.*, 2005). However, a combination of biomarkers may be needed to provide a complete characterisation of the treatment effects (beneficial and harmful) or disease progression (Lesko and Atkinson, Jr., 2001).

In this review we have summarised the characteristics of the currently available animal models of Parkinson's disease, followed by a discussion on *in vivo* monitoring techniques to obtain information on the target site distribution (PK). Here, the emphasis is on blood-brain barrier (BBB) functionality in Parkinson's disease and on the effects of Parkinson's disease on drug transport into and out of the brain, and the within brain distribution to the target site. Furthermore, to be able to determine the effect (PD) of drugs on the Parkinsonian behaviour in animal models, we have summarised the most commonly used behavioural tests used. Subsequently, the main concepts of mechanism-based PK-PD modelling are presented and discussed.

2. Animal Models of Parkinson's Disease

Animal models are "experimental preparations developed in one species for the purpose of studying phenomena occurring in another species" (McKinney, 1984). Suitable research models display clear face validity (isomorphism), predictive validity (pharmacological correlation), and construct validity (homology and similarity in the underlying neurobiological mechanisms) (Fuchs and Fliugge, 2006). In the case of animal models of Parkinson's disease, the model should reproduce the main characteristics of the human disease, such as (1) selective lesion of dopaminergic neurons that evolves over time; (2) depletion of dopamine from the striatum; (3) presence of LBs in the remaining dopaminergic neurons, and (4) easily detectable motor deficits (Bohlen Und, 2005; Yuan *et al.*, 2005). Currently applied animal models for Parkinson's disease can be separated in toxin-induced models and genetic animal models (Meredith *et al.*, 2008).

Toxin-induced animal models

A common feature of all neurotoxin-induced models is that they affect mitochondrial function, either by inhibiting mitochondrial complex I or complex III (see Figure. 1) (Schober, 2004). The mechanisms through which systemic dysfunction of complex I produces neurotoxicity are as yet unknown (Uversky, 2004).

This review gives an overview of the commonly used neurotoxins in models for Parkinson's disease, namely 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine (MPTP), paraquat, maneb and rotenone, as well as a number of less-known neurotoxins like reserpine, methamphetamine (METH) and proteosome inhibitors (PSI). A summary of the neurotoxin-induced models is given in Table 1.

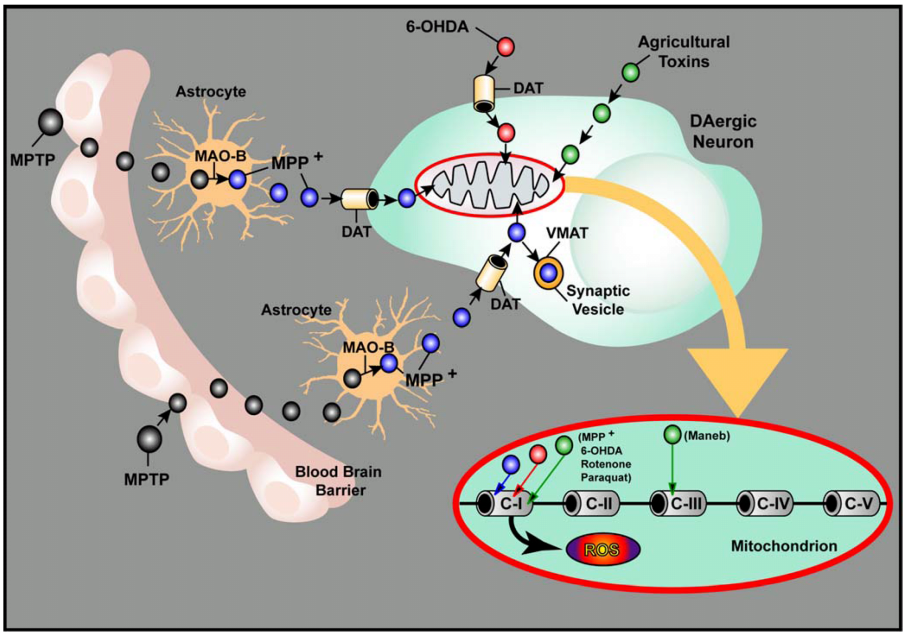

Figure 1: *Schematic overview of molecular and intracellular pathways of dopaminergic neurotoxins applied in animal models of Parkinson's disease. (taken with permission from Schober et al, 2004; Cell TissueRes 318:215-224)*

Reserpine

The administration of reserpine in rabbits was the one of the first animal models of Parkinson's disease. It caused a temporary slowness in movement that was reversed by the administration of L-DOPA (Carlsson *et al*., 1957). Reserpine is a naturally occurring alkaloid and when administered systemically to rodents, it is able to deplete dopamine at the nerve terminals and induce a hypokinetic state in the animals. However, since the effect of reserpine on the dopaminergic cells is temporary and striatal reserpine administration does not induce morphological changes in the nigral dopaminergic neurons, the model has limited use. Reserpine doesn't replicate the extensive biochemical and pathological changes seen in Parkinson's disease (Betarbet *et al*., 2002).

Methamphetamine

Methamphetamine (METH) is a psychostimulant which causes oxidative damage of nigrostriatal dopaminergic terminals (LaVoie and Hastings, 1999). In addition, METH-induced neurotoxicity may be linked to the glutamate and nitric oxide

systems within the striatum as it has been shown that glutamate antagonists are able to prevent METH-induced dopamine depletion (Kita *et al*., 2003; Sonsalla *et al*., 1989; Zeevalk *et al*., 1994). METH is an indirect catecholamine agonist, causing dopamine and norepinephrine release, and a potent inhibitor of MAO leading to an overall increase in cathecholaminergic activity in the brain (Green *et al*., 1992; Kita *et al*., 2003). METH presents an opportunity for modeling Parkinson's disease in that the neuropathology does overlap in terms of dopamine depletion, decreased tyrosine hydroxylase activity, loss of dopamine uptake sites and the loss of cell bodies in the SNc. However, there are no LBs present and striatal damage occurs in both the dopaminergic and serotonergic systems (Kita *et al*., 2003).

Table 1: *Neurotoxin-induced animal models of Parkinson's disease*

Neurotoxin	animal	How does it work?	LB	Acute/ Chronic	Applicable studies
Reserpine	Rat, mouse, rabbit	Systemic administration temporarily depletes DA. However, no morphological changes in SNc-DA neurons and it doesn't replicate typical biochemical and pathological changes.	NO	Acute	Effects of symptomatic therapies
METH	Rat, mouse, guinea pig, monkey	Oxidative damage of nigrostriatal DA terminals; DA and NE release and MAO inhibition leading to overall increase in brain CA. However, striatal damage occurs in both the dopaminergic and serotonergic systems.	NO	Acute	Mechanisms of pathogenesis
PSI	Rat, mouse	Striatal DA depletion and DA cell death with apoptosis and inflammation in the SNc. However data are not readily reproducible.	YES	Acute	Model under development
6-OHDA	Rat, mouse, monkey, cat	Selective DA cell death by oxidative stress and inhibition of mitochondrial complex I and IV. However, anterior olfactory structures, lower brain stem areas or the locus coeruleus are not affected.	NO	Acute (Chronic after intrastriatal injection)	Effects of symptomatic or neurorestorative therapies (neurprotective therapies with chronic model)
MPTP	Nonhuman primate, mouse, dog, cat, rat,	MPTP is converted to MPP+ which inhibits complex I in SNc-DA neurons. However, α-synuclein neurons in SNc were unlike typical LBs found in Parkinson's disease.	YES	Acute (Subchronic mouse model)	(1) Effects of symptomatic, neuroprotective, neurorestorative therapies (2) Mechanisms of pathogenesis
Paraquat/ Maneb	Rat, mouse	Reduces striatal TH and DA transporter immunoreactivity, striatal TH protein levels, and TH immunoreactivity and cell counts in the SNc. α-synuclein-positive inclusions in SNc neurons.	(YES)	Acute Subchronic	(1) Effects of symptomatic, neuroprotective, neurorestorative therapies (2) Role of environmental toxins in etiology
Rotenone	Rat, mouse	Rotenone denegerates SNc-DA neurons by inhibiting complex I and produces α-synuclein-positive LB-like inclusion. However, variable results and severe peripheral toxicity upon systemic administration.	YES	Chronic	(1) Effects of symptomatic, neuroprotective, neurorestorative therapies (2) Role of environmental toxins in etiology (3) mechanisms of pathogenesis

LB: Lewy Body; METH: Methamphetamine; PSI: Proteosome inhibitor; 6-OHDA: 6-Hydroxydopamine; MPTP: 1-methyl-4-phenyl-1,2,3,6-tertahydropyridine DA: dopamine; NE: norepinephrine; CA: cathecholamine

Proteosomal inhibitors

A relatively new rodent toxin-induced model in adult rats has been proposed that uses systemic administration of proteasomal inhibitors (PSI) such as epoxomicin. Animals treated with proteosomal inhibitors have been shown to develop behavioural symptoms like bradykinesia, rigidity, tremor, and an abnormal posture, which improved upon apomorphine treatment. Also, striatal dopamine depletion and dopaminergic cell death with apoptosis and inflammation in the

SNc has been observed. In addition, α-synuclein/ubiquitin-containing inclusions resembling LBs were present in some of the remaining neurons (McNaught *et al.*, 2004). Since the publication by McNaught, several laboratories have tried to reproduce these results; some groups with success, in rats or mice (Schapira *et al.*, 2006; Sun *et al.*, 2006; Zeng *et al.*, 2006), others have unfortunately failed (Bove *et al.*, 2006; Hirst and Ferger, 2008; Kadoguchi *et al.*, 2008; Kordower *et al.*, 2006; Landau *et al.*, 2007; Manning-Bog *et al.*, 2006; Mathur *et al.*, 2007). There could be various factors explaining these discrepancies between laboratories, i.e. manufacturing, storage, preparation and administration of PSI or animal-specific differences in the metabolism of the toxin (Hirst and Ferger, 2008; McNaught and Olanow, 2006). Furthermore, not much is known about the plasma and brain kinetics of PSI's (Hirst and Ferger, 2008). Conceptually the application of PSIs might be a good PD model, as proteosomal inhibition could work slowly and progressively like the disease. A prerequisite is to and a small molecule PSI (ideally for systemic administration), which enables a significant and effective proteasome inhibition in the nigrostriatal system (Hirst and Ferger, 2008).

6-OHDA

6-OHDA is the first animal model of Parkinson's disease associated with SNc dopaminergic neuronal death (Ungerstedt, 1968). 6-OHDA-induced pathology differs from Parkinson's disease as its induced toxicity is relatively selective for monoaminergic neurons, resulting from preferential uptake by dopaminergic and noradrenergic transporters (Luthman *et al.*, 1989). Other brain areas involved in Parkinson's disease, such as the anterior olfactory structures, lower brain stem areas or the locus coeruleus are not affected by 6-OHDA (Betarbet *et al.*, 2002; Del Tredici *et al.*, 2002). Inside neurons, 6-OHDA accumulates in the cytosol, where it induces cell death through oxidative stress and inhibition of mitochondrial complex I and IV (Figure 1) (Jeon *et al.*, 1995). As 6-OHDA is not able to cross the BBB, it must be administered by local stereotaxic injection into the SNc, median forebrain bundle (MFB; which carries ascending dopaminergic and serotonergic projections to the forebrain), or the striatum to target the nigrostriatal dopaminergic pathway (Dauer and Przedborski, 2003; Javoy *et al.*, 1976). Different models have been developed in mainly rodents and small monkeys (marmosets) using 6-OHDA, most of them unilaterally, resulting in a model that is commonly referred to as the "hemiparkinson model". At least in mice, rats, cats and primates, 6-OHDA is a highly effective toxin for dopaminergic neurons (Beal, 2001). Bilaterally affected animals cope with adipsia, aphagia and high mortality and

require intensive nursing care. In the hemiparkinson model, the contralateral side serves as an internal control, albeit that this side may also be influenced by the lesion, as is reflected in changes of striatal peptide and dopamine concentrations or changes in the electrical activity of neurons located in the subthalamic nucleus (Nieoullon *et al.*, 1977; Perier *et al.*, 2000; Salin *et al.*, 1996). Unilateral 6-OHDA injections into the SNc or the MFB cause an anterograde degeneration of the whole nigrostriatal dopaminergic system (O'Neill *et al.*, 2004; Sachs and Jonsson, 1975; Ungerstedt, 1968). Dopaminergic neurons start degenerating within 12-24 hr and die without apoptotic morphology. Striatal dopamine levels are depleted 2-3 days later (Faull and Laverty, 1969; Jeon *et al.*, 1995). After intrastriatal injection of 6-OHDA, it is taken up into the striatal terminals and causes a slow, progressive, induced neuron death, retrogradely, which lasts for 1-3 weeks (Murray *et al.*, 2003; Przedborski *et al.*, 1995; Sauer and Oertel, 1994). The magnitude of the lesion depends on the amount of 6-OHDA injected, the site of the injection, and the species used (Betarbet *et al.*, 2002). So far, none of the modes of 6-OHDA intoxication have led to the formation of LB-like inclusions (Dauer and Przedborski, 2003).

MPTP

MPTP currently represents the most important and most frequently used Parkinsonian toxin applied in animal models (Beal, 2001). MPTP is the byproduct of the unlawful manufacture of a synthetic meperidine derivative. Drug addicts who took MPTP accidentally developed a syndrome that clinically and pathologically resembled Parkinson's disease. After repeated injection of MPTP intravenously, chronic and severe parkinsonism developed, which was L-DOPA responsive. A tremor which was indistinguishable from the characteristic resting tremor occurred and neuropathological examination revealed moderate to severe depletion of pigmented nerve cells in the SNc, but an absence of LBs (Langston *et al.*, 1983). MPTP, which is a pro-toxin and highly lipophilic, crosses the BBB and is converted to 1-methyl-4-phenyl-2,3-dihydropyridium (MPDP) in non-dopaminergic cells (especially in astrocytes and serotonergic neurons) by MAO-B and then oxidixed to 1-methyl-4-phenylpyridinium (MPP+). Released from these cells, MPP+ is then actively transported into dopamine neurons where it impairs mitochondrial respiration by inhibiting complex I of the electron transport chain (Nicklas *et al.*, 1985; Vila and Przedborski, 2003). Endothelial cells in the microvasculature that make up the BBB contain MAO. Several studies have correlated levels of MAO with MPTP-induced neuronal loss (Kalaria *et al.*, 1987;

Riachi *et al*., 1988). Since MPP+ cannot be transported through the BBB (Riachi *et al*., 1990), the BBB can be a first line of defense against exogenous agents (Smeyne and Jackson-Lewis, 2005).
MPTP is mainly applied in nonhuman primates and in mice but also in several other species such as dog, cat, rat and goldfish (Bohlen Und, 2005). With regard to the species used, several distinct routes of MPTP administration have been established such as oral or stereotactical injection, but also systemic administration by injection (e.g. subcutaneous, intravenous, intraperitoneal or intramuscular; (Przedborski *et al*., 2001)) which is still the most common and reproducible form.

In contrast to primates, rodents are less sensitive to MPTP toxicity but the MPTP mouse model provides the most useful animal model of PD to study neuropathological and neurochemical changes (Schmidt and Ferger, 2001). In mice, systemic or intracranial administration of MPTP can result in severe damage of the nigrostriatal dopaminergic system, including symptoms such as akinesia, rigidity, tremor, gait and posture abnormalities. In MPTP-injected mice, a dramatic loss of dopaminergic neurons has been detected leading to impaired dopaminergic neurotransmission, which is accompanied by a loss of dopaminergic nerve terminals in the striatum and a dramatic reduction in striatal dopamine levels. The cell loss in the SNc is accompanied by an increase in the number of α-synuclein-immunoreactive neurons located in this brain area and an increase in α-synuclein mRNA (Vila *et al*., 2000). However, when MPTP-treated animals developed α-synuclein inclusion bodies, these were not conform the distinctive architecture of LBs in Parkinson's disease (Maries *et al*., 2003). Due to the insensitivity for MPTP, mice require relatively high doses to induce a significant loss of dopaminergic neurons resulting generally in acute pathology. Nevertheless, acute, low doses of MPTP are administered to mice to study compensatory mechanisms (Schmidt and Ferger, 2001). An acute treatment with a medial dose of MPTP induces a rapid dopaminergic degeneration with predominantly necrotic cell death (Schober, 2004) but for a subchronic induction of Parkinson's disease, medial doses of MPTP are injected 1-2 times a day for at least 5 days and for progressive chronic Parkinson's disease MPTP is given once daily at low doses over a time period of 20 days. The chronic MPTP-mouse protocol mirrors most closely the pattern of progression assumed to be that of Parkinson's disease and appears useful (Bezard *et al*., 1997). Different mouse strains react very differently to MPTP. Systemic MPTP treatment of C57BL/6 and

BALB/c mice indicated a higher susceptibility to MTPT toxicity in C57BL/6 mice. No difference between gender was observed (Sedelis *et al*., 2000).

Rats are relatively resistant to MPTP (Terzioglu and Galter, 2008). Only injections of high doses of MPTP in rats which are therapeutically pretreated, e.g. with guanethidine, to prevent peripheral catecholamine release and extensive mortality, cause significant dopaminergic neurodegeneration. Rats are therefore not recommended for MPTP studies (Giovanni *et al*., 1994).

The MPTP monkey model remains the gold standard for the preclinical evaluation of new symptomatic therapies for Parkinson's disease (Dauer and Przedborski, 2003). The most commonly used administration mode in monkeys are multiple intraperitoneal or intramuscular injections as well as intracarotid infusions (Schober, 2004). In the past, primates were nearly always treated with high doses of MPTP to induce an acute and severe degeneration of dopaminergic neurons. However, views have changed and currently, monkeys are treated more and more with low doses of the neurotoxin for a prolonged period of time resulting for a more chronic degeneration and thereby mirroring the human pathogenesis more appropriately (Przedborski *et al*., 2001). Unilateral intracarotid artery (ICA) administration of MPTP and subsequent intravenous injections of MPTP in rhesus monkeys induces an advanced stable Parkinsonian syndrome, in which the ICA injection of MPTP initiates the Parkinsonian syndrome primarily in one hemisphere and the subsequent iv. doses (administered as needed) further deplete the dopamine system to induce a bilateral lesion in a shorter period of time, with fewer side effects (Oiwa *et al*., 2003). Although MPTP is able to mimick the features of Parkinson's disease well, other brain areas involved in Parkinson's disease, such as the locus coeruleus are not affected (Dauer and Przedborski, 2003). Moreover, continuous MPTP infusion by minipumps has been reported to induce development of intracellular inclusion bodies in baboons (Kowall *et al*., 2000), but these inclusions are not similar to the LBs typically found in human disease (Maries *et al*., 2003).

Paraquat and maneb

Exposure to environmental toxins has been associated with an increased risk for developing Parkinson's disease (Chade *et al*., 2006; Tanner, 1989). The herbicide paraquat (N,N′-dimethyl-4-4′-bipiridinium) and the fungicide maneb (manganese ethylenebisdithiocarbamate) are two pesticides which have shown to induce dopaminergic neuron degeneration in the SNc. Paraquat is structurally similar to MPTP, but has no selectivity for the dopamine transporter and therefore does not

accumulate in dopamine neurons after systemic administration. It does not easily penetrate the BBB but is possibly taken up into the brain by the L-neutral amino acid transport system, then transported into striatal, possibly neuronal, cells in a Na+-dependent manner (Shimizu *et al.*, 2001). Pretreatment of paraquat-treated mice with *L*-valine or *L*-phenylalanine seems to prevent neurodegeneration completely and thus confirms the uptake of paraquat into the brain by the neutral amino acid transporter (McCormack and DiMonte, 2003). Paraquat induces a specific, although modest, dose-dependent loss of striatal dopaminergic nerve fibres and SNc neural cell bodies (Brooks *et al.*, 1999; Terzioglu and Galter, 2008), although evidence in literature seems contradictory (McCormack *et al.*, 2002; Thiruchelvam *et al.*, 2000a; Thiruchelvam *et al.*, 2000b). The toxicity of paraquat appears to be mediated by the formation of superoxide radicals and the activation of cholinergic and glutamatergic transmission (Corasaniti *et al.*, 1998; Dauer and Przedborski, 2003). Subchronic treatment of rats with systemic paraquat significantly decreases brain dopamine content in the striatum and slightly in the midbrain and cortex and stimulates glutamate efflux from neural cells or inhibits the glutamate uptake system and thereby initiating a cascade of excitotoxic reactions (Shimizu *et al.*, 2003). Levels of α-synuclein were reported as elevated in both the frontal cortex and ventral midbrains. Finally α-synuclein-positive inclusions in the SNc neurons of mice treated with paraquat were observed (Manning-Bog *et al.*, 2002).

Much less is known about the mechanisms of maneb toxicity. Maneb consists of manganese and ethylene-bis-dithiocarbamate, both of these components being potentially neurotoxic. Which of the two components of maneb is responsible for the decreased locomotor activity and potentiation of the paraquat effects on the nigrostriatal pathway, remains to be clarified (Uversky, 2004). Paraquat in combination with maneb produces synergistic effects on the nigrostriatal dopamine system. Paraquat/maneb reduces striatal TH and dopamine transporter immunoreactivity, striatal TH protein levels, and TH immunoreactivity and cell counts in the SNc but not in the ventral techmental area (VTA) (Thiruchelvam *et al.*, 2000b). The effects of paraquat/maneb on the dopaminergic system are age-dependent. A greater decrease in dopamine turnover, dopamine metabolites, nigrostriatal dopaminergic neurons and striatal TH protein levels compared with younger mice as was shown in 18-month old mice compared with younger mice treated with paraquat/maneb (Thiruchelvam *et al.*, 2003). A study in 6-month-old rats also indicated an increased susceptibility of older animals to toxicity from paraquat/maneb compared to a study with

2-moth-old rats (Cicchetti *et al.*, 2005; Saint-Pierre *et al.*, 2006).
The animal models using paraquat alone or in combination with maneb are currently not applied for the evaluation of new therapies for Parkinson's disease. Thier use is limited to investigations into therelationship between exposure to environmental toxins such as paraquat and maneb and the development of Parkinson's disease in humans.

Rotenone
Another candidate for experimental work onthe role of environmental toxins in Parkinson's disease, is the pesticide, rotenone. Rotenone is the most potent member of the rotenoids, a family of natural cytotoxic compounds extracted from tropical plants and it is widely used as an insecticide and fish poison. Rotenone is highly lipophilic and readily gains access to all organs (Talpade *et al.*, 2000) and therefore it does not require the dopamine transporter to gain access to the neural interior. Rotenone binds (at the same site as MPP+) to and inhibits mitochondrial complex I, leading to a homogenous reduction of complex I activity throughout the brain. *In vitro*, subacute rotenone treatment of differentiated SH-SY5Y neuroblastoma cells resulted in death of approximately 60% of the cells. Furthermore, it appears to reproduce the Lewy neuritic changes of early Parkinson's disease pathology, although no LB inclusions were seen (Borland *et al.*, 2008). *In vivo*, the administration of low-dose intravenous or subcutaneous rotenone to rats produces selective degeneration of nigrostriatal dopaminergic neurons accompanied by α-synuclein-positive LB-like inclusions (Betarbet *et al.*, 2000; Sherer *et al.*, 2003). Rotenone can cross cell membranes and is therefore likely to affect all cells. However, rotenone seems to preferentially target dopamine neurons. These neurons are particularly sensitive to oxidative stress because of the permanent elevated level of free radicals generated by dopamine metabolism and auto-oxidation (Giasson and Lee, 2000; Olanow, 1990). However, differences were observed in rotenone vulnerability between different populations of dopaminergic neurons within the SNc, and between dopaminergic fiber projections and cell bodies (Betarbet *et al.*, 2000). In contrast to these findings, acute intoxication with rotenone seems to produce selective damage in the striatum and the globus pallidus, but spare dopaminergic neurons in the SNc (Ferrante *et al.*, 1997). The variability in the development of dopaminergic lesions in the brain of systemically administered rotenone as well as the high mortality rate have been reported previously (Betarbet *et al.*, 2000; Fleming *et al.*, 2004; Hoglinger *et al.*, 2003; Lapointe *et al.*, 2004; Zhu *et al.*, 2004). This high-variability

demonstrates the inter-individual susceptibility to rotenone, reflecting genetic differences among rats, providing insight into the interaction between environmental and genetic influences of Parkinson's disease pathogenesis (Perier *et al.*, 2003). Systemic administration of rotenone in rats seems to affect locomotor behaviour, however this can not be correlated to the degree of nigrostriatial injury seen in these animals. Most probably, changes in locomotion are a consequence of peripheral organ toxicity (Fleming *et al.*, 2004; Lapointe *et al.*, 2004;). These results were confirmed after repeated daily subcutaneous or oral doses of rotenone in C57BL/6 mice (Inden *et al.*, 2007; Richter *et al.*, 2007) although at high daily oral doses of 10 and 30 mg/kg a reduction of TH immunoreactivity in SNc was seen with variability being the lowest at the highest dose (Inden *et al.*, 2007). Interestingly, absorption of rotenone in the gastrointestinal tract is slow and incomplete and rotenone is effectively metabolised by the liver which would not favor the oral route for rotenone administration (Bove *et al.*, 2005). In another study systemic exposure of mice to rotenone demonstrated an acute increase in dopamine turnover which seems to be common to other mitochondrial toxins (Thiffault *et al.*, 2000).

It was demonstrated in a model of Drosophila melanogaster that L-DOPA is able to reverse rotenone-induced locomotor changes (Coulom and Birman, 2004) and a study with rats showed similar results whereby L-DOPA (in combination with a decarboxylase inhibitor) was able to reverse rotenone-induced catalepsy, postural changes and decreased locomotion, indicating that the behaviour was a consequence of the degeneration of dopaminergic neurons, rather than from systemic toxicity of rotenone. The rats were treated daily with low systemic doses of rotenone via i.p. administration for 60 days or rotenone was infused directly into medial forebrain bundle of the brain (Alam and Schmidt, 2004). These modes of administration cause a slow degeneration which makes it suitable to study neuroprotective agents (Schmidt and Alam, 2006).

Due to its inconsistent and unpredictable effect on the nigrostriatal pathway, the chronic (systemic) administration of rotenone is not yet suitable to become a routine animal model for Parkinson's disease (Bove *et al.*, 2005). Next to genetics (Perier *et al.*, 2003), various factors such as age (Phinney *et al.*, 2006; Richter *et al.*, 2007), rat species (Betarbet *et al.*, 2000; Schmidt and Alam, 2006) and even environmental temperature (Crutchfield and Dluzen, 2006) might contribute to the variability in results. An alternative approach is when rotenone is administered directly into the brain. Although this does not represent the natural exposure to environmental toxins, it might develop into an animal model which

could be applied in preclinical (PK-PD) studies in the search for neuroprotective strategies for Parkinson's disease.

Intracerebral-administered rotenone (2 µg dissolved in 5 µl 10% DMSO solution into the MFB) produced a decrease in dopamine and its metabolites in the striatum (about 70%) and SNc (35% for dopamine and 55% for DOPAC), without affecting the serotonin system (Antkiewicz-Michaluk *et al.*, 2004). Another study demonstrated that stereotaxic administration of rotenone into the MFB, SNc or striatum decreased dopamine levels in the striatum (96%, 62% and 30%, respectively). Furthermore, these animals showed behavioural changes to a similar degree as seen in 6-OHDA lesioned rats (Sindhu *et al.*, 2006). Intranigrally, rotenone-infused animals exhibited progressive ipsilateral rotations when challenged with amphetamine on days 7, 14, 21 and 28 whereas animals that received a MFB infusion of rotenone only displayed this behaviour on day 28 (Sindhu *et al.*, 2005). Thus, an infusion of rotenone into the MFB might develop a slower, more progressive degeneration of dopaminergic cells compared to an intranigral infusion. Moreover, the decrease in striatal dopamine on day 32 seems greater in MFB-infused animals compared with intranigrally infused animals (Sindhu *et al.*, 2005). In another study, an intranigral infusion caused a time-dependent reduction of complex-I activity, an increased production of hydroxyl radicals and a significant depletion of striatal dopamine in the ipsilateral SNc. No changes were seen in striatal serotonin levels. TH immunostaining revealed a highly significant decrease in the staining intensity in the striatum and an overt decrease in the area positive for TH in the ipsilateral SNc (Saravanan *et al.*, 2005). Bilateral infusion of rotenone into the MFB showed a strong increase in catalepsy, a decrease in locomotor activity and a significant depletion of striatal dopamine levels compared to sham-lesioned rats. L-DOPA is able to reverse the motor deficits in rats, confirming the depletion of dopaminergic neurons in these animals (Alam *et al.*, 2004). In our opinion, the intracerebral infusion of rotenone, specifically into the MFB, has the most potential as an animal model for mechanism-based PK-PD studies in search for treatments with neuroprotective properties.

Genetic animal models

The majority of Parkinson's disease cases (>95%) are sporadic, although some genes (associated with the PARK loci) have been identified and linked to rare forms of Parkinson's disease. Currently, there are six clearly defined genetic

causes of Parkinson's disease. There are α-synuclein (PARK1) and LRRK2 (PARK8), which result in autosomal dominant Parkinson's disease and Parkin (PARK2), DJ-1 (PARK7) and PINK1 (PARK6), which result in autosomal recessive Parkinson's disease (Pankratz and Foroud, 2007). The gene UCHL1 (PARK5) has been implicated but not confirmed.

For Parkin, DJ1 and PINK1, all of which cause early-onset Parkinson's disease, genetic mouse models can easily be made by null mutation of such genes (knockout mice). For the dominantly inherited gain-of-function mutations such as in α-synuclein (PARK1) and LRRK2 (PARK8), transgenic mouse models have been created in which extra copies of the gene are introduced into the mouse genome or delivered by lenti- or adeno-associated virus (Terzioglu and Galter, 2008). Since α-synuclein is a major component of LBs, and mutations in α-synuclein may result in nigrostriatal dopaminergic degeneration in familial Parkinson's disease it has gained the most focus in the development of transgenic mice or Drosophila flies, which express the wild-type or mutated α-synuclein. Transgenic mice overexpressing α-synuclein or expressing mutated forms of α-synuclein display a number of features seen in Parkinson's disease such as progressive accumulation of α-synuclein-and ubiquitin-immunoreactive inclusions in neurons in the neocortex, hippocampus, and SNc, mitochondrial DNA damage and degeneration, loss of dopaminergic terminals in the basal ganglia and motor impairments (Martin *et al.*, 2006; Masliah *et al.*, 2000). Similar results were found in rats overexpressing α-synuclein (Mochizuki *et al.*, 2006). Interestingly, several lines of α-synuclein null mice have a complete or partial resistance to MPTP (Dauer *et al.*, 2002; Drolet *et al.*, 2004; Schluter *et al.*, 2003). Animal models based on the transgenic expression of wild-type and mutated α-synuclein do not demonstrate all of the key features of Parkinson's disease, like a massive loss of dopamine nigrostriatal neurons (Betarbet *et al.*, 2002; Fleming *et al.*, 2005). Rapid neurodegeneration is observed after viral transduction but is limited to the targeted region and does not mimic the broad pathology observed in the disease (Chesselet, 2008). Nevertheless, they provide an important opportunity to study the involvement of α-synuclein in Parkinson's disease pathogenesis.

Other genetic models include the Parkin knockout mice, DJ-1 knockout mice, NURR1 and PITX3-APHAKIA, among others. The reader is referred to some excellent reviews for a more detailed description of these models (Fleming *et al.*, 2005; Terzioglu and Galter, 2008).

3. Measuring Target Site Distribution and BBB Functionality in Animal Models of Parkinson's Disease

The distribution of drug molecules between plasma and the target site in the brain is a crucial factor in the effects of drugs used in the treatment of Parkinson's disease. In the previous chapter it has been outlined that multiple mechanisms are involved in the distribution of drug molecules to the target site in the brain (i.e. brain perfusion, transport across the BBB, distribution within the brain). Moreover it has been demostrated that the functionality of the BBB may change under disease conditions. This underscores the need of studying the brain distribution kinetics of drugs used in the treatment of Parkinson's disease. In this pragraph we discuss principles and applications of intracebral microdialysis as a tool monitor the time course of extracellular brain concentrations.

Intracerebral microdialysis involves the insertion of a microdialysis probe into a selected area of the brain. The probe, consisting of a hollow tube and a semi-permeable membrane, is constantly perfused with a physiological solution. During perfusion, substances around the semipermeable part of the probe will diffuse from higher to lower concentration into the dialysate (Figure 2).

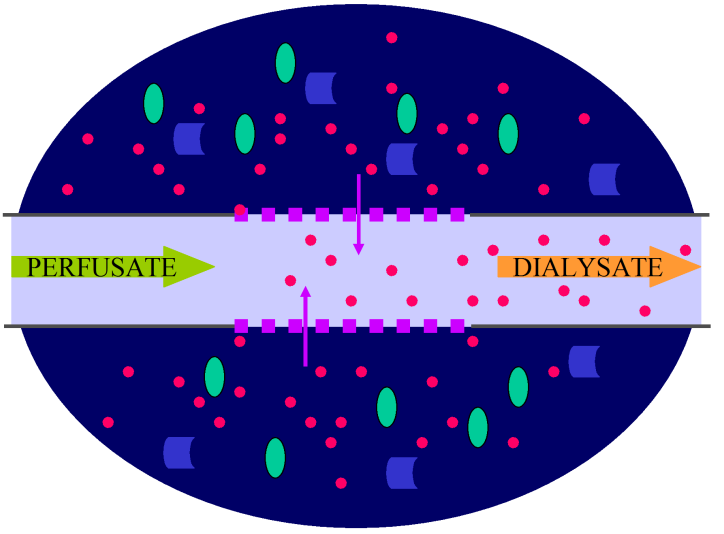

Figure 2: The principle of microdialysis

Drug concentrations in brain dialysate reflect but do not equal free (unbound) concentrations in brain ECF due to the constant flow of perfusion fluid (de Lange

et al., 1999). In vitro recovery, or better, in vivo recovery methods such as retrodialysis, no-net-flux and dynamic-no-net-flux need to be applied to be able to relate the concentration in the dialysate to the true concentration in the ECF (Bouw and Hammarlund-Udenaes, 1998; de Lange *et al.*, 1997; de Lange *et al.*, 1999).

Many studies using intracerebral microdialysis in experimental animals have already shown its usefulness and special value in monitoring brain pharmacokinetics. It has been shown that this technique providies better estimates of BBB transport parameters compared with the more classical tissue homogenate-pharmacokinetic approach (de Lange *et al.*, 1997; de Lange *et al.*, 1998; Hammarlund-Udenaes *et al.*, 1997; Hammarlund-Udenaes, 2000; Sawchuk and Elmquist, 2000; Wang and Welty, 1996; Xie *et al.*, 1999). A prerequisite for this application is that BBB transport characteristics are not significantly influenced by the microdialysis probe implantation and its presence in the brain. This was an initial concern but based on a series of studies performed to validate intracerebral microdialysis in the characterisation of passive, as well as active, BBB transport (de Lange *et al.*, 1994; de Lange *et al.*, 1995a; de Lange *et al.*, 1995b; de Lange *et al.*, 1998; de Lange *et al.*, 2000; Malhotra *et al.*, 1994; Ooie *et al.*, 1997; Wang and Welty, 1996), it has been demonstrated that this prerequisite holds, provided that the technique is used under well-controlled surgical and experimental conditions (de Lange *et al.*, 1997).

Intracerebral microdialysis is not only able to measure kinetics of exogenous compounds but also offers the possibility of monitoring endogenous compounds such as neurotransmitters and any changes in their kinetics as a consequence of a disease and/or its treatment (Baskaya *et al.*, 1997; Carter *et al.*, 1995; Frantz *et al.*, 2002; Fuchs and Hauber, 2003; Glick *et al.*, 1994; Hashiguti *et al.*, 1993; Jonkers *et al.*, 2001; Kaakkola and Wurtman, 1992; Napolitano *et al.*, 2003; Souza Silva *et al.*, 1997). Specifically in Parkinson's disease, intracerebral microdialysis has been applied to study the kinetics of L-DOPA and its metabolites in healthy and diseased rodents (Cannazza *et al.*, 2005; Fedrowitz *et al.*, 2000; Gerin, 2002; Giovanni *et al.*, 1994; Holmer *et al.*, 2005; Kannari *et al.*, 2006; Kostrzewa *et al.*, 2005; Marti *et al.*, 2000; Sarre *et al.*, 1992) or monkeys (Alexander *et al.*, 1994; Zhang *et al.*, 2003). Using intracerebral microdialysis, one is able to measure the PK of a (new) drug for Parkinson's disease and determine its distinct brain penetration properties and consequently measure its effect (PD) on brain neurotransmitters. Simultaneously, intracerebral microdialysis is able to measure a biomarker or a combination of biomarkers specific for Parkinson's disease in the same animal.

Furthermore, by means of dual probe microdialysis, one is able to measure compounds in different brain areas, in the same rat, at the same time (Galeffi *et al.*, 2003; Pudovkina *et al.*, 2002; Westerink *et al.*, 1998). Using mechanism-based PK-PD disease progression modeling, presumed neuroprotective properties of new treatments for Parkinson's disease can thus be investigated in a preclinical setting.

4. Measuring Behaviour and Drug Effects in Animal Models of Parkinson's Disease

To be able to assess lesion-induced disability and/or treatment-induced recovery, the appropriate but simple testing routines should be chosen. Behavioural tests should reflect histological and/or functional deficits or preservation of the injured tissue (Yuan *et al.*, 2005). Animal models of Parkinson's disease can be either unilateral as well as bilateral, requiring different behavioural assessments. The following section summarises behavioural tests which are commonly used in animal models of Parkinson's disease.

Rotometry

Rotational behaviour of unilateral-lesioned animals in response to dopamine receptor agonists has been the conventional method in the assessment of dopamine-mediated responses since the 1970s (Ungerstedt and Arbuthnott, 1970). Unilateral lesions (mainly by 6-OHDA) produce a hemiparkinsonian syndrome, which includes asymmetries of body posture, and contralateral sensorimotor deficits (Cenci *et al.*, 2002). The rotometry test consists of recording the number of turns that are performed by an animal after a challenge with dopamine agonists (amphetamine or apomorphine). The net rotational assymetry score is expressed as full body turns per minute (Moore *et al.*, 2001). Animals with unilateral dopamine depletion will turn contralaterally to the hemisphere in which dopamine-receptor stimulation is stronger; that is, they will turn towards the side of the lesion after challenge with dopamine-releasing drugs and away from it after treatment with L-DOPA or dopamine agonists (Yuan *et al.*, 2004).

Elevated body swing test

The elevated body swing test allows behavioural testing of unilateral lesioned animals without using drugs, to determine the natural response following a lesion (Roghani *et al.*, 2002). This test was first described by Borlongan (Borlongan and Sanberg, 1995) and adapted for various studies (Roghani *et al.*, 2002; Yuan *et al.*,

2004) including the assessment of body axis bias ('curling') (Henderson *et al*., 1999). The elevated body swing test involves elevating the animal by handling its tail and recording the frequency and direction of the swing behaviour. Unilateral nigral 6-OHDA-lesioned rats exhibited significant biased swing activity with the direction contralateral to the lesioned side, corresponding to the direction of apomorphine-induced rotations. A 30 seconds elevated body swing test was noted as the peak time for biased swing activity (Borlongan and Sanberg, 1995).

Head turning

In this test, the position of the head relative to the body axis is measured by placing the rat into a standard cage and allowing it to habituate. The position of the head (> 10 ° deviation left or right of the midline, or neutral) was noted every second for 60 seconds. The net number of seconds the head was positioned ipsilaterally minus the number of seconds positioned contralaterally is calculated over 3 min of observations (Henderson *et al*., 1999). From this, ipsilateral side bias and overall head-turning activity indices can be calculated (Moore *et al*., 2001).

Staircase test

The staircase test, also referred to in modified versions as the skilled paw reaching test, is a behavioural test that consists in reaching for food inside a special box and allows for a sensitive measure of skilled reaching by each limb in an independent manner (Montoya *et al*., 1991). This test is not only applied in unilateral-lesioned animals of Parkinson's disease, but aso in animals with experimental stroke, hypoxia and peripheral neuropathy (Pagnussat *et al*., 2009). The staircase apparatus comprises a test box from which runs a narrow corridor with a central plinth. On either side of the plinth is a descending 'staircase' of steps. Two food pellets are placed onto each step of two staircases located one on either side of the plinth. Rats are placed in the box and can reach down either side of a plinth to grasp lift and retrieve food pellets from the steps of the staircase. The numbers of pellets removed provides a quantifiable measure of the distance and efficiency of reaching skill. The design is such that the rat can only reach pellets on one staircase with its left paw and on the other with its right paw, thereby providing separate measures of performance for each limb (Jeyasingham *et al*., 2001; Kirik *et al*., 1998; Montoya *et al*., 1991; Moore *et al*., 2001; Pagnussat *et al*., 2009).

Stepping test

This test is a modification of the bracing test (Schallert *et al*., 1979). The rat is held

by the experimenter fixing its hindlimbs with one hand and the forelimb not to be monitored with the other, while the unrestrained forepaw is touching the table. The number of adjusting steps was counted while the rat is moved sideways along the table surface (90 cm in 5 s), in the forehand direction, for both forelimbs (Kirik *et al*., 1998; Olsson *et al*., 1995; Paille *et al*., 2007).

Grip strength

The grip strength test measures fore- and hindlimb grip-strength in rats or mice and is able to asses the effect of drugs, age and toxins on muscle strength (Meyer *et al*., 1979). This test was later modified to provide measurement of the two forelimbs, separately but simultaneously. A description of the apparatus is described elsewhere (Dunnett *et al*., 1998). Rats and mice naturally cling to the bars until they can no longer resist the pull, and then let go. The applied force at the point at which the rodent releases its grip for each paw is recorded by two spearate strain gauses connected to a digital readout (Jeyasingham *et al*., 2001).

Catalepsy test

Catalepsy in laboratory animals is defined as a failure to correct an externally imposed posture, so the typical catalepsy test consists of placing an animal into an unusual posture and recording the time taken to correct this posture (Sanberg *et al*., 1988). The most common catalepsy test is the 'bar test' where rats are placed with both forepaws on bars 9 cm above and parallel from the base and were in a half-rearing position. Latency time of the removal of the paw is then recorded (Nehru *et al*., 2008; Zhou *et al*., 2007). There are, however, many variations of this bar test as summarised elsewhere (Sanberg *et al*., 1988). Other tests applied to measure catalepsy are by using parallel bars, platforms or pegs to situate animals in unusual positions (Sanberg *et al*., 1988).

Rotorod

The rotorod (Rota-Rod) test, in which animals walk on a rotating drum, is widely used to assess motor status in laboratory rodents. Animals are pre-trained on the rotorod and then tested at a series of increasing rod speeds. This test is suitable for initial screening of lesions or therapies both in unilateral and bilateral lesioned animals (Rozas *et al*., 1997; Rozas and Labandeira Garcia, 1997). Performance is measured by the duration that an animal stays up on the drum as a function of drum speed. High-speed video recording methods of the animal walking on the rotorod gives information about qualitative aspects of walking movements

(Whishaw *et al*., 2008). Using the rotorod test, 6-OHDA-lesioned rats showed chronic impairment in their posture and in the use of the limbs contralateral to the lesion (Whishaw *et al*., 2003).

5. Mechanism-Based PK-PD Modelling Techniques

PK-PD modeling is increasingly applied in modern drug research. PK-PD modeling aims at characterisation and prediction of the time course of drug action in health and disease (Breimer and Danhof, 1997). In recent years PK-PD modeling has evolved from a descriptive discipline into a mechanistic science that is applied in all phases of drug discovery and development In this context advanced mechanism-based PK-PD models are now being developed, which have improved properties for *in vitro*/*in vivo*-, interspecies- and healthy volunteers to patients extrapolation and prediction.

Mechanism-based models

PK-PD has evolved from the basic concept of the dose-response relationship to sophisticated models enabling the understanding of the underlying mechanisms of drug action (Csajka and Verotta, 2006). Mechanism-based PK-PD models contain specific expression to characterise specific processes on the causal path between drug administration and response. These processes include: 1) target distribution, 2) target binding and activation, 3) transduction/homeostatic feedback and 4) disease processes/progression. As such mechanism-based PK-PD modeling utilizes concepts from physiologically-based pharmacokinetic (PBPK) modeling, receptor theory, dynamical systems analysis and disease systems analysis (Danhof *et al*., 2007). A key element of mechanism-based PK-PD modeling is the explicit distinction between the drug-specific and biological system-specific parameters. Drug-specific parameters (target affinity and target activation) can often be predicted on the basis of *in vitro* bioassays and are often identical between species and individuals, whereas biological system-specific parameters (PD interactions, time-dependent transduction mechanisms, homeostatic feedback mechanisms, disease processes and disease progression) can only be estimated by *in vivo* systems analysis and typically their values can vary between species, individuals, disease state and experimental conditions (Danhof *et al*., 2007; Danhof *et al*., 2008).

Interspecies scaling / allometry

In drug development many pharmacological and toxicological studies are performed in small laboratory animals such as mice, rats, rabbits, dogs and monkeys. Species differences and therefore interspecies scaling is an important issue for the prediction of pharmacokinetic parameters from these animals to humans. The assumption that animals have similar physiology, biochemistry and cellular structure has allowed interspecies scaling of heart rate, blood flow, blood volume and organ size (Mordenti, 1986). Clearance (Cl), V_d, and elimination half-life ($t_{1/2}$) are the three most frequently extrapolated pharmacokinetic parameters. Over the years, many approaches have been suggested to improve the prediction of these pharmacokinetic parameters in humans from animal data and consist of allometric methods (Mahmood, 1999) and PBPK models (Danhof *et al*., 2008; Rowland, 1985).

In short, the allometric approach is based on the power function Y = aWb, where the bodyweight of the species is plotted against the pharmacokinetic parameter of interest on a log-log scale. Using this approach, Cl is not predicted very well (error between predicted and observed clearance > 30%). Thus, several other approaches have been proposed, which have been described elsewhere (Feng *et al*., 2000; Fura *et al*., 2008; Mahmood and Balian, 1999; Sinha *et al*., 2008). The PBPK models provide a mechanistic-based evaluation of drug disposition in which they use a physiologically realistic compartmental structure (Danhof *et al*., 2008; De Buck *et al*., 2007) and therefore is a better approach for interspecies extrapolation as it uses species-specific data on tissue structure, volume and composition and on the associated blood flows (Danhof *et al*., 2008). The structure of the PBPK model is essentially common to all mammalian systems, thereby facilitating interspecies scaling, and these models are uniquely suited to predict tissue and organ exposure (Rowland *et al*., 2004).

Compared with the allometric approach, PBPK models also seem to have a higher percentage of successful predictions (Ito and Houston, 2005; Jones *et al*., 2006; Zuegge *et al*., 2001). PBPK modeling has been applied successfully to take species differences in functionality of transporters at the BBB into account and was used to predict target exposure of drugs such as selective serotonin re-uptake inhibitors and semi-synthetic opioids (Geldof *et al*., 2008; Groenendaal *et al*., 2007a; Groenendaal *et al*., 2007b; Liefaard *et al*., 2005).

Disease progression

In vivo systems analysis aims at the explanation of physiology and disease from the level of interacting components such as molecular pathways, regulatory networks, cells, organs, and ultimately the entire organism. In conventional PK-PD analyses, the values of these biological system-specific parameters in the absence of a drug are kept invariable with time, and physiology is generally considered constant at baseline. However, for progressive, chronic diseases like Parkinson's disease, this is not a realistic description as the biological functions (e.g. dopaminergic neurons) deteriorate over the time course of the (mostly symptomatic) treatment period. Therefore, disease progression analysis has been proposed where the influence of a drug effect on the change in disease status over time is characterised (Chan and Holford, 2001; Holford and Peace, 1992). In these first disease-progression models for clinical Parkinson's disease rating scales, the signs and/or symptoms of disease and their response to treatment are modeled directly, without consideration of the underlying biological system as only information on clinical symptoms or outcome was available.

A theoretical framework for mechanism-based disease progression models has been proposed, in which time-dependent changes in the biological system-specific parameters of diseased subjects are taken into account (Post *et al*., 2005). This is an important factor when searching for a drug treatment for Parkinson's disease intended to slow or stop the disease processes and disease progression. PK-PD modeling is increasingly applied in drug discovery and preclinical development i.e. in the selection of drug candidates with the most favorable PK and PD properties. For this purpose, the development of chronically instrumented animal models for the determination of the time course of drug concentration and effect is very important. An animal model for Parkinson's disease displaying the slow progressive nature of the disease would be able to provide the necessary information (biological system-specific parameters) needed to determine the neuroprotective properties of new drugs, preclinically. Availability of relevant biomarkers could result in a more detailed and mechanistic description of disease progression and serve as useful tools in these disease models. Within the context of mechanism-based PK-PD modeling, a biomarker is defined as a measure that characterises, in a strictly quantitative manner, a process, which is on the causal path between drug administration and effect (Danhof *et al*., 2005). However, a combination of biomarkers ("fingerprint") might be needed to provide a complete characterisation of the treatment effects (beneficial and harmful) or disease progression (Lesko and Atkinson, Jr., 2001).

6. Conclusions

As we have stated in the introduction, there is no single animal model which reflects all aspects of Parkinson's disease but one model may reflect specific aspects suitable for specific Parkinson's disease research questions. Specifically, an animal model for Parkinson's disease displaying the slow progressive nature of the disease constitutes a basis to obtain the essential biological system-specific information to determine the neuroprotective properties of new drugs, preclinically. In this review we have summarised the currently available animal models of Parkinson's disease. An animal model of Parkinson's disease should reproduce the main characteristics of the human disease, such as (1) selective lesion of dopaminergic neurons that evolves over time; (2) depletion of dopamine from the striatum; (3) presence of LBs in the remaining dopaminergic neurons, and (4) easily detectable motor deficits (Bohlen Und, 2005; Yuan *et al*., 2005).

Although all of the models display some means of damage to the nigrostriatal dopaminergic system (Table 1), causing a decrease of striatal dopamine, only the MPTP and rotenone models have shown to develop LB-like inclusion bodies. However, when MPTP-treated animals developed α-synuclein inclusion bodies, these were not conform the distinctive architecture of LBs in Parkinson's disease (Maries *et al*., 2003). MPTP, 6-OHDA and rotenone can be applied to induce a (sub)chronic disease progression. For MPTP, the chronic MPTP-mouse protocol mirrors most closely the pattern of progression assumed to be that of Parkinson's disease and appears useful (Bezard *et al*., 1997). In the case of 6-OHDA, an intrastriatal injection causes a slow, progressive, induced neuron death with the disadvantage that is is retrogradely and thus not displaying the normal disease progression which is from the SNc to the striatum and not vice versa. Given this, rotenone seems to be the best candidate for the development of an animal model for Parkinson's disease with a slow, progressive induction of the disease. Rotenone is usually administered systemically. Due to its inconsistent and unpredictable effect on the nigrostriatal pathway, the chronic (systemic) administration of rotenone is not yet suitable to become a routine animal model for Parkinson's disease (Bove *et al*., 2005). Next to genetics (Perier *et al*., 2003), various factors such as age (Phinney *et al*., 2006; Richter *et al*., 2007), rat species (Betarbet *et al*., 2000; Schmidt and Alam, 2006) and even environmental temperature (Crutchfield and Dluzen, 2006) might contribute to the variability in results. An alternative approach is when rotenone is administered directly into the brain. Although this does not represent the natural exposure to environmental toxins, it might develop into an animal model which could be applied in

preclinical (PK-PD) studies in the search for neuroprotective strategies for Parkinson's disease. Either way, rotenone was able to induce Parkinson's disease symptoms such as catalepsy, postural changes and decreased locomotion (Alam and Schmidt, 2004). These modes of administration cause a slow degeneration which makes it suitable to study neuroprotective agents (Schmidt and Alam, 2006).

An unilateral model using rotenone would create a model where the contralateral side serves as an internal control. Caution should be made, however, as this side may also be influenced by the lesion. Using intracerebral microdialysis, extracellular, unbound concentrations of endogenous compounds (e.g. biomarkers for Parkinson's disease or BBB functionality) as well as of exogenous compounds (e.g. antiparkinson drugs or marker compounds for specific transport mechanisms of the BBB) could be quantified simultaneously at various time point during the progression of the disease. Any, or a combination, of the behavioural tests may be applied for the unilateral lesioned animal model although one should always take the influence of a microdialysis probe on the behaviour into consideration, if applicable.

A bilateral model using rotenone (administration either systemically or intrecerebrally) would give a more realistic representation of the human disease progression and behaviours. A separate control (sham-lesioned) group of animals would be needed for comparison of $brain_{ECF}$ concentrations of endogenous or exogenous compounds as well as for the behavioural experiments. In the latter case, not all of the summarised tests as described here, can be applied. The grip strength test, the catalepsy test and the rotorod test are suitable for use in the bilateral animal model. The staircase test may be applied to determine the difference in the total amount of food pellets taken between control and lesioned rats, irrespective of which paw was used. Similarly with the stepping test, where the number of total adjusting steps could be counted between control and lesioned rats, again, irrespective of which paw was used.

The data obtained from these experiments give information on processes on the causal path between drug administration and response (e.g. target site distribution, target binding and activation, transduction/homeostatic feedback and diseases processes/progression). With this, mechanism-based PK-PD models can be developed which can have properties for extrapolation and prediction in systems pharmacology research (Danhof *et al*., 2007). Systems pharmacology aims at the development of an understanding of the interactions between pathophysiology and drug action. To date most PK-PD modeling research has

focused on healthy systems. The use of a chronic animal model for Parkinson's disease is a first step in the characterisation of drug effects on disease processes and disease progression. The next particularly intriguing challenge is the prediction of drug effects on disease progression in man from this preclinical research.

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Section II :
The Rotenone Rat Model of Parkinson's Disease

Chapter 4

The Exploration of Rotenone as a Toxin for Inducing Parkinson's Disease in Rats

Application in BBB Transport and PK-PD Experiments

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Abstract

In search for a suitable rat model to study alterations in blood-brain barrier (BBB) transport mechanisms in the course of Parkinson's disease progression, experiments were performed to characterise Parkinson's disease and safety markers following subcutaneous (SC) and intracerebral (IC) infusion of the toxin rotenone in the rat. Studies were performed using male Lewis rats. SC infusion of rotenone (3 mg/kg/day) was performed via an osmotic minipump. IC infusion of rotenone occurred directly into the right medial forebrain bundle (MFB) at three different dosages. At different times following rotenone infusion, behaviour, histopathology (tyrosine hydroxylase (TH) and α-synuclein immunocyto-chemistry), peripheral organ pathology (adrenals, heart, kidney, liver, lung, spleen and stomach) were assessed. In part of the SC and IC rats, BBB transport profiles of the permeability marker sodium fluorescein were determined using microdialysis. SC rotenone failed to produce dopaminergic lesions and led to extensive peripheral organ toxicity. BBB permeability for fluorescein following SC rotenone was changed due peripheral toxicity. In contrast, IC rotenone produced a progressive lesion of the nigrostriatal dopaminergic pathway over 28 days with no associated peripheral toxicity. IC rotenone also exhibited a large increase in amphetamine induced rotational behaviour. In addition, a few IC rats showed α-synuclein immunoreactivity and aggregation. Following IC rotenone, no changes in passive BBB permeability were detected after 14 days. SC rotenone only produced peripheral toxicity affecting BBB permeability. IC rotenone appeared to create a progressive lesion of the rat nigrostrial pathway, and may therefore be a more appropriate model of Parkinson's disease progression, compared with the most commonly used 6-OHDA rat model.

1. Introduction

Parkinson's disease is a chronic, progressive neurodegenerative disease in which nigrostratial dopaminergic neurons are gradually lost, leading to a decrease in dopamine concentration in the striatum (Dauer and Przedborski, 2003). It is the second most common neurodegenerative disease and its current therapy focuses mainly on symptomatic treatment by replacing the loss of dopamine in the striatum (Mercuri and Bernardi, 2005) by L-DOPA, the dopamine precursor. The main goal for future treatment of Parkinson's disease is the discovery and development of neuroprotective/neurotrophic drugs to diminish or, better, to halt the disease progression (Bonuccelli and Del Dotto, 2006; Chen and Le, 2006).

An important consideration for the development of neuroprotective drugs for the treatment of Parkinson's disease is the relationship between disease progression and the pharmacokinetic and pharmacodynamic (PK-PD) properties of the drug. In the PK-PD relationship of anti-Parkinsonian drugs a number of factors are of importance. One factor is transport of the drug across BBB. Drug transport between blood and the brain is governed by a number of BBB transport mechanisms as described in **Chapter 2** of this thesis.
BBB functionality is dynamically controlled by blood components and the surrounding brain cells by direct contact or indirectly by their extracellular products. Thus, BBB functionality may vary among different physiologic, pathologic, and chronic drug treatment conditions, and this may affect the BBB transport of the drug.

It is important to reveal in which direction and to what extent the different BBB transport mechanisms (and actual BBB transport of a drug) are influenced by Parkinson's disease progression. This should be adressed in a systematic manner, in relation to the physico-chemical properties of the drug. To that end preclinical studies are needed using an adequate animal model of Parkinson's disease in which Parkinson's disease progression can be identified. The most widely used animal models of Parkinson's disease are neurotoxin models with MPTP (mice, cats and primates) or 6-OHDA (mice, rats, cats and primates) (**Chapter3**, Beal, 2001). These models are generally acute (Betarbet *et al.*, 2002; Schober, 2004), but in some cases MPTP subchronic models in both primates and mice have been used (Schober, 2004). Recently, a mouse Parkinson's disease model was introduced in which MPTP was continuously administered through an osmotic minipump (Fornai *et al.*, 2005). This models appears to have the advantage over the other MPTP models that there is formation of inclusion bodies in the SNc. Although these data look very promising, it has not yet been described elsewhere.
An intrastriatal injection of 6-OHDA seems to also show a more progressive degeneration of nigral dopaminergic cells compared to intranigral injections or injections into the MFB (Sauer and Oertel, 1994; Cicchetti *et al.*, 2002). However, no Lewy body formation has yet been observed in any of the 6-OHDA models. Other approaches, which have extensively been reviewed elsewhere (Betarbet *et al.*, 2002; Uversky, 2004) include the genetic models of Parkinson's disease and the use of reserpine, methamphetamine, 3-nitrotyrosine paraquat in combination with maneb and rotenone to induce Parkinson-like symptoms. The SC infusion of rotenone to induce Parkinson's disease in the rat neurons has been reported, in

Table 1: *Setup of the experiments performed for the subcutaneous and intracerebral rotenone models. In the experiments for the subcutaneous model a 3 mg/kg/day dose of rotenone was used. In the experiments for the intracerebral rotenone model, three doses were used unless otherwise indicated: 0.5 µg, 2.0 µg and 5.0 µg.*

		Subcutaneous rotenone model				Intracerebral rotenone model			
SALINE		number of rats (n=)				number of rats (n=)			
	frequency of measurement	**Day 4**	**Day 14**	**Day 21**	**Day 28**	**Day 4**	**Day 7**	**Day 14**	**Day 28**
bodyweight	(week)daily until day of sacrifice	24	18	12	6	96	72	48	24
behaviour (Rotarod)	(week)daily until day of sacrifice	24	18	12	6			NA	
histology (striatum & SNc)	once on day of sacrifice	6	6	6	6	24[d]	24[d]	24[d]	24[d]
behaviour (amphetamine challenged rotations)[a]	not measured for saline			NA				NA	
BBB permeability (fluorescein)[a]	once on day of sacrifice		3					NA	
peripheral organ pathology	once on day of sacrifice		10[a]		10[a]	24[d]	24[d]	24[d]	24[d]
VEHICLE (DMSO/PEG)		number of rats (n=)				number of rats (n=)			
	frequency of measurement	**Day 4**	**Day 14**	**Day 21**	**Day 28**	**Day 4**	**Day 7**	**Day 14**	**Day 28**
bodyweight	(week)daily until day of sacrifice	24	18	12	6	96	72	48	24
behaviour (Rotarod)	(week)daily until day of sacrifice	24	18	12	6			NA	
histology (striatum & SNc)	once on day of sacrifice	6	6	6	6	24[d]	24[d]	24[d]	24[d]
behaviour (amphetamine challenged rotations)[a]	once, 7 days prior to sacrifice (Day 28)			NA				8	
BBB permeability (fluorescein)[a]	once on day of sacrifice		3				8		
peripheral organ pathology	once on day of sacrifice		10[a]		10[a]	24[d]	24[d]	24[d]	24[d]
ROTENONE		number of rats (n=)				number of rats (n=)			
	frequency of measurement	**Day 4**	**Day 14**	**Day 21**	**Day 28**	**Day 4**	**Day 7**	**Day 14**	**Day 28**
bodyweight	(week)daily until day of sacrifice	60	45	30	15	96	72	48	24
behaviour (Rotarod)	(week)daily until day of sacrifice	60	45	30	15			NA	
histology (striatum & SNc)	once on day of sacrifice	15	15	15	15	24[d]	24[d]	24[d]	24[d]
behaviour (amphetamine challenged rotations)[a]	once, 7 days prior to sacrifice (Day 28)			NA				12[c]	
BBB permeability (fluorescein)[a]	once on day of sacrifice		7				12[c]		
peripheral organ pathology	once on day of sacrifice		10[a,b]		10[a,b]	24[d]	24[d]	24[d]	24[d]

[a]histology results on the brains of these rats are not presented in this paper, [b]a total of 4 rats died spontaneous during these experiments and were not included in the analysis, [c]only 5 µg rotenone was used in these experiments, [d]total of 24 rats; 8 per treatment group, NA: not applicable

which a gradual increase in striatal dopaminergic denervation with associated α-synuclein-positive cytoplasmic inclusions in nigral neurons (Betarbet *et al.*, 2000; Sherer *et al.*, 2003). Most recently, the IC infusion of rotenone into the MFB or SNc has been reported to affect the nigrostriatal pathway and produce deficits which were reversed by L-DOPA (Alam *et al.*, 2004; Antkiewicz-Michaluk *et al.*, 2004; Saravanan *et al.*, 2005; Sindhu *et al.*, 2005).

In this study, we have first investigated the influences of SC administration of rotenone (3 mg/kg/day) via an osmotic minipump in the rat on bodyweight and behaviour (locomotor activity) as measured frequently within a period of 4 weeks. Within this period, at different time intervals, rats were sacrificed and brains were used to assess nigrostriatal damage based on immunohistological staining on tyrosine hydroxylase, while in addition for all rats peripheral organ pathology was determined. In a separate experiment, at 14 days of SC administration of rotenone, BBB permeability was assessed using sodium fluorescein as a marker. In a last experiment, peripheral organ pathology was performed to determine the effect of 3 mg/kg/day rotenone systemically. In a subsequent series of experiments, the influence of IC administration of rotenone in the MFB at three different doses of rotenone (0.5, 2.0 and 5.0 µg) on nigrostriatal damage (immunohistological staining on tyrosine hydroxylase) was determined. To be able to compare these results to the results obtained in the experiments using the subcutaneous rotenone model, we carefully monitored bodyweight and peripheral organ pathology was also performed on these rats. After confirming we were producing a suitable lesion we used the highest rotenone dose (5.0 µg) in behavioural experiments and in a microdialysis experiment using sodium fluorescein as a BBB permeability marker. A summary of all the experiments which are presented in this paper is shown in Table 1.

2. Materials and Methods

Statement on use and care of animals

The experiments described in this paper were approved by the Ethical Committee on Animal Experimentation of the University of Leiden (DEC numbers 118 and 5069). For all experiments (Table 1) inbred adult male Lewis rats (250-300 g, Charles River BV, Maastricht, The Netherlands) were used. The rats were housed in standard plastic cages (six per cage before surgery and individually after surgery) with a normal 12-hour day/night schedule (lights on 7:30 AM) and a temperature of 21°C. The animals had access to standard laboratory chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum.

Surgical methods

Placement of osmotic minipumps

In these experiments, the rats were inplanted with an Alzet osmotic minipump (2ML-4, Alzet CR, Maastricht, The Netherlands) placed subcutaneously on the back of the rat. For the rats in the microdialysis experiment, the pump was placed 7 days after microdialysis surgery. The osmotic minipump was filled with either 3mg/kg/day Rotenone (Pestanal®, Sigma Alldrich BV, Zwijndrecht, the Netherlands) dissolved in a 1:1-mixture of dimethylsulfoxide (DMSO, Sigma Alldrich BV, Zwijndrecht, the Netherlands) with polyethylene glycol (PEG 200, Sigma Alldrich BV, Zwijndrecht, the Netherlands) as the vehicle (DMSO:PEG; 1:1), or filled with the vehicle alone or saline (0.9% NaCl).

Pumps were incubated in sterile saline at 37 °C overnight. For the implantation, rats were deeply anesthetised using isoflurane (Forene®, Abbott B.V., Hoofddorp, The Netherlands). The Alzet osmotic minipumps were implanted under the skin on the back of the rat. The rats were weighed on regular basis to monitor general well-being. Rats were euthanised either after 7, 14, 21 or 28 days or after the development of hunched posture suggestive of abdominal pain and upon inadequate feeding or grooming, suggestive of ongoing toxicity.

Implantation of blood cannulas

The surgery for the microdialysis study was performed under anesthesia with an intramuscular injection of 0.1 mg/kg medetomidine hydrochloride (Domitor 1 mg/ml, Pfizer, Capelle a/d IJssel, The Netherlands) and 1 mg/kg ketamine base (Ketalar 50 mg/ml, Parke-Davis, Hoofddorp, The Netherlands). Two indwelling cannulae (pyrogen-free, nonsterile polyethylene tubing, Portex Limited) were implanted, one in the left femoral artery and one in the left femoral vein. The

cannula in the left femoral vein was used for administration of fluorescein, whereas the cannula in the left femoral artery was used for serial collection of arterial blood samples. The cannulae were tunneled subcutaneously and fixed at the back of the neck with a rubber ring. The skin in the neck was stitched with normal sutures. The skin in the groin was closed with wound clips.
To prevent clotting and cannula obstruction, the cannulae were filled with a 25% (w/v) polyvinylpyrrolidone solution (PVP; Brocacef, Maarssen, The Netherlands) in pyrogen-free physiological saline (B. Braun Melsungen AG, Melsungen, Germany) containing 20 IU/ml heparin (Hospital Pharmacy, Leiden University Medical Center, Leiden, The Netherlands). After the implantation of the cannulae, the rats were placed in a stereotaxic frame and the skull was exposed for brain surgery.

Microdialysis surgery for the subcutaneous model
After the implantation of the cannulas, a small hole was drilled to allow the implantation of a microdialysis guide cannula (CMA/12, Aurora Borealis Control B.V. Schoonebeek, The Netherlands) in the striatum (relative to bregma (Paxinos *et al.*, 1985): AP: +0.4; L: +3.2; V: -3.5). Two support screws were placed to hold the guide, which was glued to the skull with dental acrylic cement (Howmedia simplex rapid + methylacrylate, Drijfhout, Amsterdam, The Netherlands). The osmotic minipump was placed 7 days after the microdialysis surgery and the experiment was carried out 14 days after pump implantation.

Microdialysis surgery for the intracerebral model
After the implantation of the cannulas, the rats received a unilateral infusion into the right MFB (AP: -2.8; L: +2.0; V: -9.0 relative to bregma; (Paxinos *et al.*, 1985)) at a rate of 0.1 µl/min for 30 minutes of either vehicle (DMSO/PEG, 1:1; n=8; referred to as sham) or 5.0 µg of rotenone (n=12). After the infusion, the needle was kept in place for another 5 minutes to allow diffusion of the fluid without leakage along the track of the needle. Subsequently, two small holes were drilled into the skull to allow implantation of a microdialysis guide cannula (CMA/12, Aurora Borealis Control B.V. Schoonebeek, The Netherlands) in the left and in the right striatum relative to bregma (AP: +0.4; L: +/-3.2; V: -3.5, relative to bregma; (Paxinos *et al.*, 1985)).
One support screw was placed as an extra anchor for fixation of the guide, which was glued to the skull with dental acrylic cement (Howmedia simplex rapid + methylacrylate, Drijfhout, Amsterdam, The Netherlands). The microdialysis

experiment was carried out 14 days after microdialysis surgery.

Experimental methods

Subcutaneous model: Behavioural experiment

For this study (in total n=108), the rats received an Alzet osmotic minipump filled with either rotenone in vehicle (n=60, for a dose of 3mg/kg/day), vehicle alone (n=24), or saline (n=24). For each treatment, the rats were divided into 4 groups with different treatment durations of respectively 4, 14, 21 and 28 days after which the rats were given an overdose of Nembutal® (Natriumpentobarbital 60 mg/ml, Ceva Sante Animale, Naaldwijk, The Netherlands) and the thorax was opened and the rat was perfused with 30 ml of saline followed by 30 ml of 10% phosphate buffered formalin (pH=7.0) via the left ventricle of the heart. Brains were removed for histopathology. During the entire course of the experiment, the bodyweight of the rats was monitored daily from Monday to Friday (Table 1). Before implantation of the minipump, the rats were trained daily for two weeks on the Rotarod (Ugo Basile, Comerio, Italy). After implantation, the rats' ability to stay on the Rotarod was tested on a daily basis during the week, in two sessions. Before the first session the rat was weighed, and then placed on the drum of the Rotarod with the head facing in the direction opposite to the direction of rotation. This forced the rat to move forward in order to stay on the rotating drum. The drum was started at a speed of 2 rotations per minute (r.p.m) and accelerated every 30 seconds to a final maximum speed of 20 r.p.m. after 5 minutes. At this time, the session was ended and the rat was allowed to recover for a 30 minute period before the second session was started. As a behavioural read-out, the time that the rat was able to stay on the drum was recorded. All rats that were unable to remain on the Rotarod for at least 250 seconds before the start of the treatment were excluded from data analysis.

Intracerebral model: Behavioural experiment

For this study, male Lewis rats received a unilateral infusion into the MFB of either DMSO/PEG (1:1; n=8; vehicle) or 5.0 μg of rotenone (n=12). Approximately 21 days after rotenone infusion the animals were placed in automated rotometers (Med. Associates Ltd.). The apparatus consisted of perspex bowls where each rat was linked to a harness carrying an infrared sensor at the top connected to a computer with ROTORAT software. The animals were tested for rotations in absence (baseline) and in the presence (stimulation) of amphetamine (5 mg/kg i.p.) to evaluate the effects of rotenone treatment on stimulant-induced rotations.

On the day of sacrifice (day 28), the animals were given an overdose of anaesthetic and the thorax was opened and perfused with 30 ml of saline followed by 30 ml of 10% phosphate buffered formalin (pH 7.0) via the left ventricle of the heart. Brains were removed for histopathology.

Microdialysis experiment

At 18-24 hours prior to the experiment, the microdialysis probes (CMA12, membrane length of 4.0 mm; Aurora Borealis Control B.V. Schoonebeek, the Netherlands) were gently inserted into the guide cannulas after removal of the probe dummies.

The microdialysis experiment was started between 7:00 and 8:00 a.m. The inlets of both microdialysis probes were connected by FEP tubing (fluorinated ethylene propylene tubing; internal volume of 1.2 µL/100 mm length; Aurora Borealis Control B.V. Schoonebeek, the Netherlands) to syringe pumps (Beehive, Bas Technicol, Congleton, United Kingdom). The probes were perfused with aECF (composition in mM: NaCl 145; KCl 2.7; CaCl2 1.2; MgCl2 1.0; ascorbic acid 0.2 in a 2 mM phosphate buffer pH 7.4 (Moghaddam and Bunney, 1989) at a flow rate of 2 µl/min. The outlets consisted of fused silica tubing (I.D. 150 µm, O.D. 375 µm; Composite Metal Services Ltd, Iikley, United Kingdom) and were connected to a microsample collector (Univentor 820; Antec, Leiden, The Netherlands), in which the samples were collected and cooled (4^{o}C). After a stabilisation period of 60 minutes, the *in vivo* recovery of fluorescein was determined by the retrodialysis method. For this purpose, the probes were first perfused with a fluorescein solution (10 or 20 ng/ml in aECF) for 60 minutes to collect 6 fractions. The relative loss of fluorescein was calculated as an average of this period. After this period, the syringes were switched to blank aECF for the washout phase of 90 minutes. After the washout period, the intravenous administration of fluorescein was started. The venous cannula was connected to a syringe containing fluorescein (Sigma Alldrich BV, Zwijndrecht, the Netherlands) in saline (0.9% NaCl). For a dose of 6 mg/kg of fluorescein, a 2-minute intravenous infusion was started at a rate of 50 µl/min. In the first 120 minutes of the experiment, microdialysis fractions were collected at 10-minute intervals and from 120-180 minutes and from 180-240 minutes at 20- and 30-minute intervals, respectively. Blood samples (50 µl in heparinised Eppendorf vials) were taken at 0, 0.5, 2, 4, 6, 8, 10, 15, 20, 25, 30, 45, 60, 90, 120, 180 and 240 minutes after start of the fluorescein infusion. The blood samples were centrifuged for 10 minutes at 5000 r.p.m. and the plasma was pipetted into Eppendorf vials. All samples were stored at -20 C.

Histopathology

TH immunohistochemistry was performed to quantify the degree of dopaminergic neurodegeneration. In short, the brains were cut twice into 6-mm segments using a rodent brain matrix, processed, and embedded in paraffin wax. Coronal sections (8 μm) were cut through the striatum (at 1.2 mm caudal to bregma) and the SNc (from -4.5 to -6.2 mm caudal to bregma) (Paxinos *et al.*, 1985) with a sledge microtome (Microm, Walldorf, Germany). The sections were deparaffinised and rehydrated and endogenous peroxidase was quenched with 0.3% H_2O_2 for 30 min. The slides were placed in pepsin (0.2 g of Sigma-p-7000 pepsin in 50 ml of 0.01 M HCl) for 30 min, washed, and non-specific binding was blocked with 1.5% normal goat serum (Vectastain rabbit IgG ABC kit; Vector Laboratories, Burlingame, CA). This was followed by the application of the primary rabbit polyclonal anti-tyrosine hydroxylase antibody (AB152 incubated for 18 h at room temperature; Chemicon International, Temecula, CA), the secondary biotinylated antibody (Vectastain rabbit IgG ABC kit for 30 min) and the horseradish peroxidase conjugate (Vectastain rabbit IgG ABC kit for 30 min). Visualisation was carried out using 3,3'-diaminobenzidine (Vector SK-4100 Vector Laboratories, Burlingame, CA) as a chromogen. The slides were coverslipped using DPX mountant. Adjacent nigral sections were immunostained for α-synuclein using an anti-α-synuclein (1:200, AB15530, Abcam, UK).

After staining, striatal sections were scanned using SprintScan 35 with PathScan Enabler™ at a resolution of 1012 dpi. Optimas 5.2 software was used to measure the optical density of manually-defined areas of each black and white image produced as a mean grey value (MGV). TH-IR was measured for the slide (background), cortex (control tissue staining), corpus callosum (control non-cellular staining), dorsal striatum (caudate putamen, CPu) and ventral striatum (nucleus accumbens, NAcc). The values were adjusted for non-specific staining by subtracting the MGV of the corpus callosum or cortex, an area that should have no specific TH-staining. Values obtained were entered into a spreadsheet sorted by treatment group, and the mean, standard deviation and standard error (sem) calculated using formulas. These means and errors were subsequently plotted and unless otherwise stated, all striatal TH-IR values are corrected for cortical TH-IR. A series of sections through the SN (-4.5 mm -6.2 mm) were evaluated and stained. After careful evaluation under the microcope the TH-positive cells within the SNc were counted at one selected stereotaxic level (-5.2 to 5.4 mm caudal to bregma) in both vehicle treated and the rotenone-lesioned animals. The

cells were counted manually using a digital camera (Hitachi HV-C20A) attached to a light microscope (LEICA DMLB, Leica Microsystem Wetzlar GmbH) at ×20 magnification and the lesioned side is expressed as a percentage of the intact side. Light microscopy was also used to provide a qualitative assessment of the presence of α-synuclein in the SNc in the intracerebral experiment.

Peripheral organ pathology

The pathological evaluation of the brain and peripheral organs of the subcutaneous experiment was performed on 56 rats and of the intracerebral experiment on 96 rats (Table 1). After 7, 14, 21 or 28 days of treatment (for the subcutaneous experiment only 14 or 28 days), the animals were given an overdose of Nembutal® (Natriumpentobarbital 60 mg/ml, Ceva Sante Animale, Naaldwijk, The Netherlands) and the thorax was opened and the rat was perfused with 30 ml of saline followed by 30 ml of 10% phosphate buffered formalin (pH=7.0) via the left ventricle of the heart. Adrenals, heart, kidney, liver, lung, spleen and stomach were removed and immersed in 10% neutral buffered formalin (pH=7.0), trimmed after adequate fixation according to a standard operating procedure inspired from the Registry of Industrial Toxicology Animal-data (RITA) standards (Bahnemann *et al.*, 1995), and appropriate samples routinely processed to tissue blocks embedded with paraffin wax. Tissue sections were cut at approximately 3 µm, stained with Harris' hematoxylin and eosin (HE), Oil red O for neutral fat and Von Kossa's stain for mineral/calcium deposits and examined by light microscopy for relevant changes by a pathologist, without knowledge of the treatment status.

Analysis of Fluorescein

The plasma and microdialysate samples obtained in the subctaneous model experiment were analysed for fluorescein concentrations by HPLC with fluorescence detection. The HPLC system comprised a PSS Suprema size exclusion column (8x300 mm, 10 µm particle size), with a mobile phase of 0.05 M ammoniumacetate (pH=9.0) and acetonitrile (4:1 v/v). The flow rate was maintained at a flow of 1.5 ml/min. The detection was performed using a fluorescence detector (LC240, Perkin Elmer, United Kingdom) with the excitation wavelength set at 488 nm and the emission wavelength set at 512 nm. The microdialysate samples (15 µl) were injected directly onto the HPLC column. For the analysis of the plasma samples, 10 µl of plasma was added to 990 µl of an icecold artificial extracellular fluid (described above) and vortexed for 10 seconds. A 50 µl aliquot of this mixture was injected directly onto the HPLC system

equipped with a refill guard column (2 mm I.D. x 20 mm) (Upchurch Scientific, Oak Harbor, WA, USA) packed with C18 (particle size 20-40 µm) (Alltech, Breda, The Netherlands).

For the analysis of fluorescein in plasma and microdialysate samples from the intracerebral experiment, we developed an assay using the Fluostar Optima, which was a fast and reliable method. The results of the validation of this assay are presented in Table 2. The detection limit was 0.08 ng/ml. For the analysis of the microdialysate samples, 10 µl microdialysate was pipetted onto a black 96-well plate (FIA-plate flat bottom/medium binding, Greiner, Alphen a/d Rijn, The Netherlands) and 40 µl of a 0.25 M NaOH solution was added. For the analysis of the plasma samples, an aliquot of 10 µl plasma was pipetted onto a black 96-well plate and 90 µl of a 0.1 M NaOH solution was added. The fluorescence intensity was measured on the Fluostar Optima (BMG Labtech, Offenburg, Germany) at the emission wavelength of 530 nm and the excitation wavelength of 480 nm.

Table 2: *Validation of the determination of fluorescein: intra-assay and inter-assay variability, coefficients of variability and accuracy*

Compound	Added	Intra-assay (n=4)			Inter-assay (n=10)		
		Found (mean ± SEM)	CV	Accuracy	Found (mean ± SEM)	CV	Accuracy
	(ng/mL)	(ng/mL)	(%)	(%)	(ng/mL)	(%)	(%)
Fluorescein	1.0	0.9 ± 0.03	6.0	93.7	1.1 ± 0.08	22.3	107.0
	2.0	2.0 ± 0.02	1.6	98.8	2.0 ± 0.03	4.6	102.1
	5.0	5.1 ± 0.03	1.1	101.7	4.9 ± 0.07	4.4	97.5
	10.0	10.1 ± 0.08	1.7	101.3	10.3 ± 0.22	6.9	102.7

Statistical analysis

All data were statistically evaluated for significance by one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparison test except for the microdialysis fluorescein data from the subcutaneous experiment which were evaluated using Student's t-test due to the small sample size of the saline and vehicle groups. Values of $P<0.05$ were considered significant.

3. Results

Subcutaneous model: bodyweight and locomotor activity

The subcutaneous infusion of rotenone produced a time-related reduction ($P<0.001$) in bodyweight of rotenone treated rats, compared with vehicle- or saline-treated rats (Figure 1). This reduction was most pronounced in the first 10-14 days following the start of the treatment. Behavioural analysis using the Rotarod was performed at various time points after infusion of saline, vehicle or rotenone (Figure 2A). The analysis was based on the time-period in which the rat was able to remain on the Rotarod (latency time). Results were categorised into 3 panels; panel 1 for latency times ranging between 0 and 100 sec, for panel 2 between 100 and 200 sec, and for panel 3 between 200 and 300 sec. In saline-treated rats and vehicle-treated rats only 2-3% of the animals had a Rotarod latency lower than 100 seconds whereas in rotenone-treated rats this number had increased to 30% (panels A1, B1, C1). Also, 77% of the saline-treated rats and 86% of the vehicle treated rats were able to stay on the apparatus for more than 200

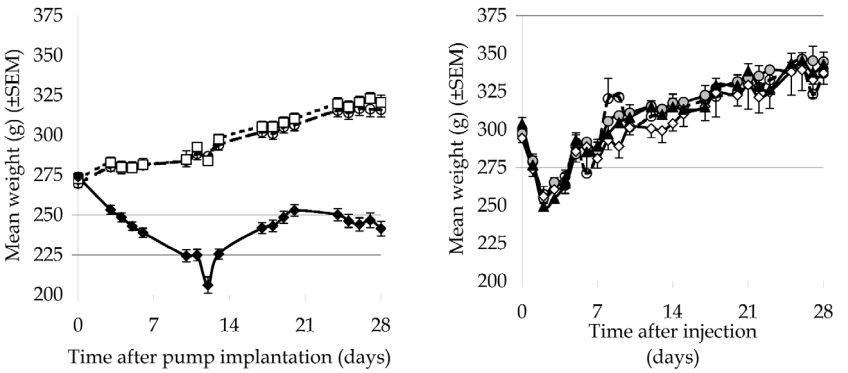


Figure 1: *(LEFT panel) The effect of the subcutaneous infusion of (o) saline, (□) vehicle (DMSO/PEG) and (•) 3.0 mg/kg/day rotenone on bodyweight over time. The data are expressed as mean values ±SEM. Statistical analysis (one-way ANOVA) showed no significant difference in weight between all treatment groups on day 0 ($P>0.05$) and no significant change between the saline and vehicle groups for any time point ($P>0.05$). Significant differences were observed between the rotenone treated group and the saline treated group ($P<0.001$) or vehicle treated group ($P<0.001$) for all time points. (RIGHT panel) The effect of an intracerebral infusion of (o) artificial ECF, (• light grey) 0.5 µg rotenone, (◊) 2.0 µg rotenone or (▲) 5.0 µg rotenone ($n=24$) on the bodyweight over time. The data are mean values±SEM. Statistical analysis (one-way ANOVA) showed no significant difference between all groups.*

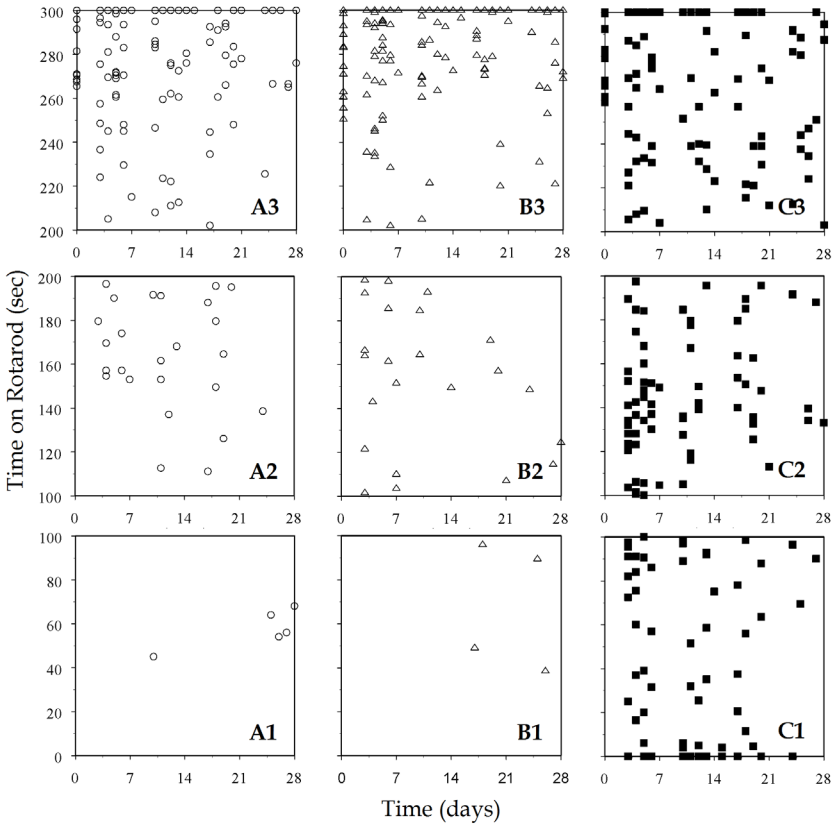


Figure 2A: *Behaviour in the subcutaneous rotenone model: The time that a rat was able to remain walking on the Rotarod at different days following implantation of the osmotic minipump in (o) saline treated rats, (△) vehicle (DMSO/PEG)-treated rats, (■) 3.0 mg/kg/day rotenone-treated rats. Panels A1-C1 represent the amount of data points in the 0-100 seconds region. Panels A2-C2 represent the amount of data points in the 100-200 seconds region. Panels A3-C3 respresent the amount of data points in the 200-300 second region.*

seconds, but only 45% of the rotenone-treated rats were able to stay on the Rotarod for that period of time (panels A3, B3, C3). Therefore, rotenone-treatment impaired Rotarod performance, with a greater percentage of these animals falling off the apparatus at early time points and fewer animals staying on for the full testing period (300 seconds).

Intracerebral model: bodyweight and rotational behaviour

The effect of the intracerebral infusion of either aECF, 0.5 µg rotenone, 2.0 µg rotenone or 5.0 µg rotenone into the MFB on the bodyweight of the rat (Figure 1) indicated that was a decrease in bodyweight of about 10-15% in all treatment groups in the first 2 days after surgery. However, from 2 days onwards until the end of the experiment (day 28) the bodyweight increased as normal in all treatment groups and overall there were no statistically significant differences among the various treatment groups. In agreement with this, the experimenter's observations of the rats with regard to grooming indicated no noticeable differences among control rats and rotenone treated rats. To determine the behavioural consequences of intracerebral infusion of rotenone, we performed an experiment in which we measured amphetamine (5 mg/kg i.p.) induced rotations in rats infused with 5.0 µg of rotenone and compared these results to sham (=DMSO/PEG; 1:1) infused rats (Figure 2B). The left panel depicts the mean total rotation score and the right panel illustrates the time course of the rotations for both the sham infused rats and the rotenone infused rats. The baseline of both groups, measured before the injection of amphetamine, shows no preference of any group for a particular side. However, after the amphetamine challenge the rotenone infused had a large and significant increase in the number of ipsiversive rotations.

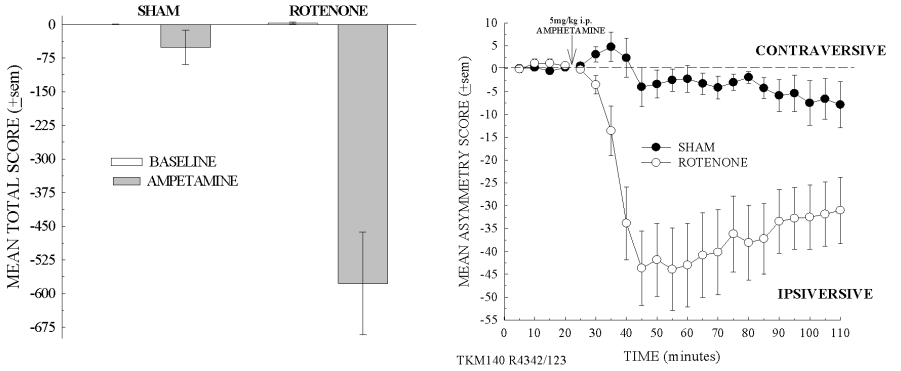


Figure 2B: *Behaviour in the intracerebral rotenone model: Amphetamine (5 mg/kg i.p.) induced rotations measured on day 21 after infusion of either sham (DMSO/PEG; 1:1) or 5µg of rotenone into the MFB. Left panel depicts the mean total rotation score (±SEM) after amphetamine injection and the right panel shows the mean asymmetry score over time after amphetamine injection.*

Table 3: *The effect of a subcutaneous infusion of saline (n=6 per timepoint), vehicle (n=6 per timepoint) or 3 mg/kg/day of rotenone (n=15 per timepoint) on the intensity of the striatal TH-immunoreactivity. The effect of different doses of rotenone (n=8 per dose and per timepoint) and artificial ECF (n=8 per timepoint) infused into the MBF on the percentage (lesioned vs. intact brain side) striatal TH immunostaining in time.*

STRIATUM					
		4 days (MGV)	**14 days** (MGV)	**21 days** (MGV)	**28 days** (MGV)
Subcutaneous Rotenone model	Saline	104 ± 7	103 ± 11	85 ± 20	102 ± 11
	Vehicle	99 ± 10	98 ± 13	73 ± 11	103 ± 10
	Rotenone	88 ± 6	96 ± 7	89 ± 7	95 ± 6
		4 days (%)	**7 days** (%)	**14 days** (%)	**28 days** (%)
Intracerebral Rotenone model	aECF	93 ± 3	91 ± 3	96 ± 4	89 ± 3
	0.5 µg Rotenone	60 ± 12	64 ± 8	55 ± 13	64 ± 10
	2.0 µg Rotenone	50 ± 13	34 ± 15	42 ± 11	98 ± 2
	5.0 µg Rotenone	70 ± 13	44 ± 9	27 ± 9	35 ± 12

The data are presented as Mean ± SEM; MGV: Mean Gray Value

Table 4: *The effect of a subcutaneous infusion of saline (n=6 per timepoint), vehicle (n=6 per timepoint) or 3 mg/kg/day of rotenone (n=15 per timepoint) on the nigral cell count. The effect of different doses of rotenone (n=8 per dose and per timepoint) and artificial ECF(n=8 per timepoint) infused into the MBF on the percentage (lesioned vs. intact brain side) nigral cell count in time.*

SUBSTANTIA NIGRA					
		4 days (cell count)	**14 days** (cell count)	**21 days** (cell count)	**28 days** (cell count)
Subcutaneous Rotenone model	Saline	163 ± 18	155 ± 13	204 ± 18	146 ± 8
	Vehicle	137 ± 21	136 ± 43	135 ± 38	131 ± 9
	Rotenone	129 ± 9	157 ± 17	145 ± 11	141 ± 8
		4 days (%)	**7 days** (%)	**14 days** (%)	**28 days** (%)
Intracerebral Rotenone model	aECF	104 ± 9	101 ± 7	84 ± 9	90 ± 5
	0.5 µg Rotenone	68 ± 15	92 ± 9	62 ± 9	62 ± 6
	2.0 µg Rotenone	72 ± 13	45 ± 12	68 ± 2	91 ± 6
	5.0 µg Rotenone	63 ± 13	74 ± 7	58 ± 6	35 ± 8

The data are presented as Mean ± SEM.

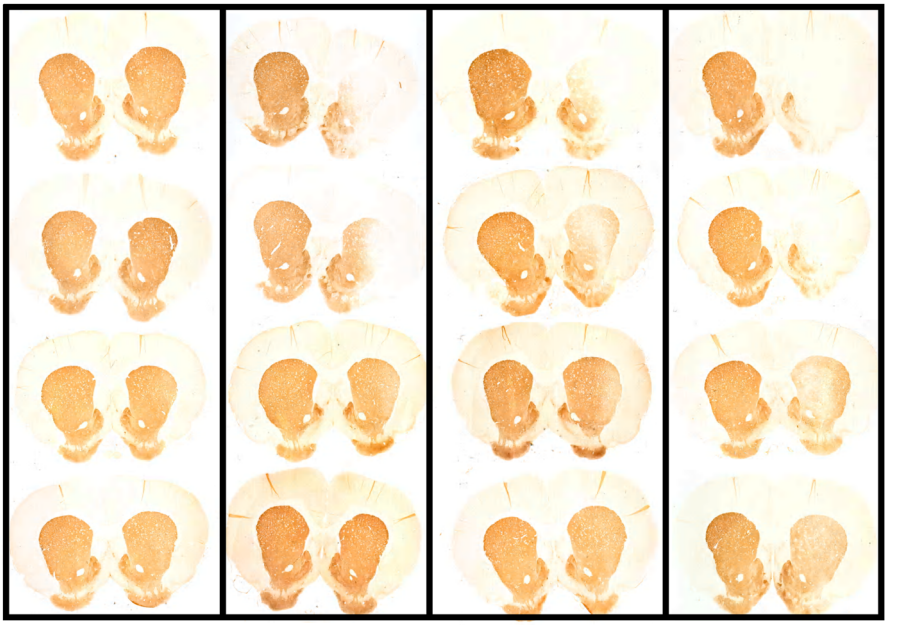


Figure 3A: *Intracerebral rotenone model: Images of tyrosine hydroxylase immunostaining of coronal sections taken at the level of the striatum from aECF or rotenone-infused rats (0.5, 2.0 or 5.0 µg) taken 14 days after infusion. Each treatment group is represented by 4 rats, the left striatum represents the intact side of the animal and the right striatum represents the lesioned side of the same animal. The lowest (0.5 µg) and middle (2.0 µg) rotenone doses show partial dopaminergic denervation whereas the highest rotenone dose (5.0 µg) had more pronounced loss in staining and some rats had almost complete dopaminergic denervation.*

Histopathology

Subcutaneous model

Looking at the individual subcutaneous rotenone rat model data from the behavioural and microdialysis experiments, only 2% of the rotenone-treated rats showed any loss of TH staining in the striatum. The loss of striatal staining in these particular rats showed a similar pattern to that reported by Betarbet (Betarbet *et al.*, 2000). No loss of cell bodies in the SNc of any animals in the present studies was observed. The mean data for the striatum and SNc are shown in Table 3 and Table 4, respectively. No significant difference was seen in the mean striatal TH immunostaining of control, vehicle and rotenone treated rats or in the mean number of TH positive cells in the SNc among these groups.

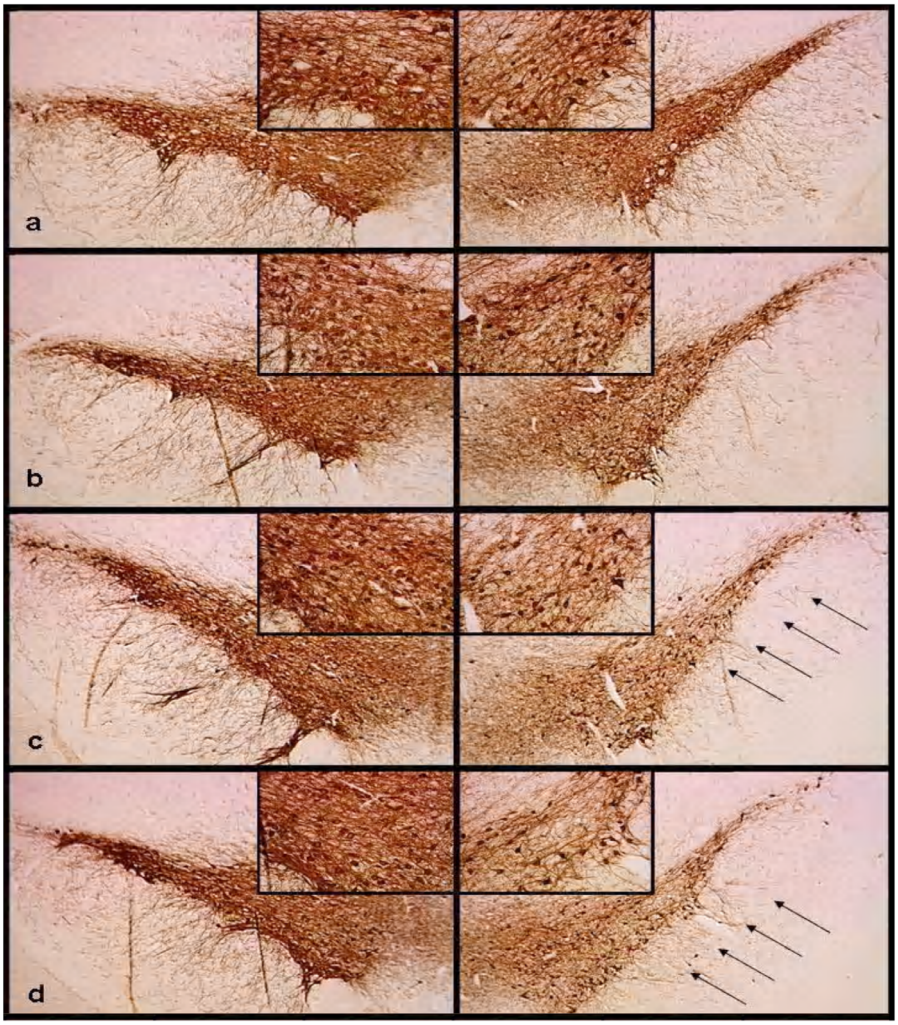


Figure 3B: *Intracerebral rotenone model: Images of tyrosine hydoxylase staining at the level of the substantia nigra from aECF (**a**) or rotenone-infused rats (0.5 (**b**), 2.0 (**c**) or 5.0 (**d**) µg) on day 14 after infusion. The left hand images show the intact substantia nigra with higher magnification of the cell bodies as insets. The right hand side shows the lesioned side of the brain with insets showing a higher magnification of the cell bodies. There is a clear dose dependent loss in TH positive cells and processes (see arrows) with increasing concentrations of rotenone.*

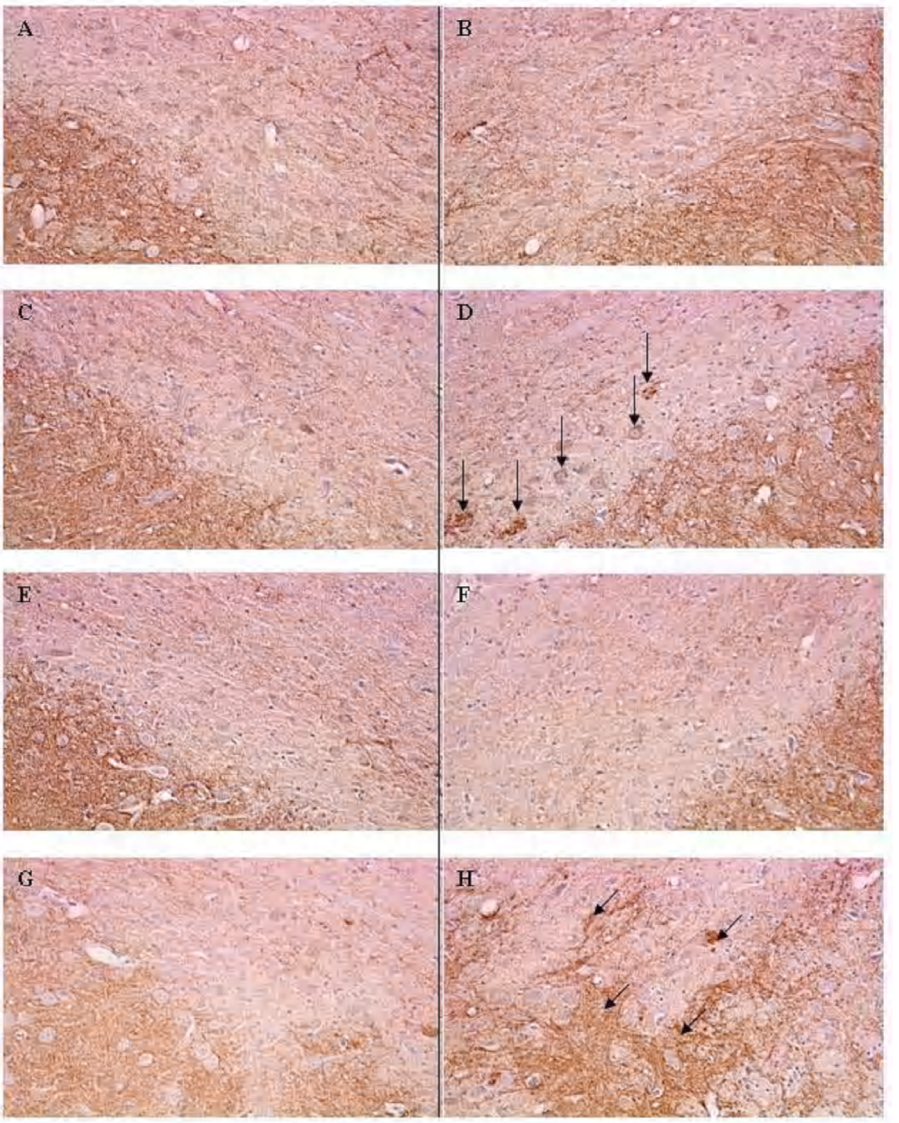


Figure 4: *Intracerebral rotenone model: Examples of α-synuclein positive staining in the SNc of rats that have received 0.5 µg (**C** and **D**), 2.0 µg (**E** and **F**) or 5.0 µg (**G** and **H**) rotenone or aECF (**A** and **B**) into the left medial forebrain bundle (MFB, stereotaxic coordinates: AP: -2.8, Lat: +2.0, V: -9.0) and killed off 28 days post surgery. Both the intact SNc (**A**, **C**, **E** and **G**) and the lesioned SNc (**B**, **D**, **F** and **H**) are shown at 20x magnification. α-synuclein staining was visualised with 3-6-diaminobenzidine (DAB) with a haematoxylin counter stain.*

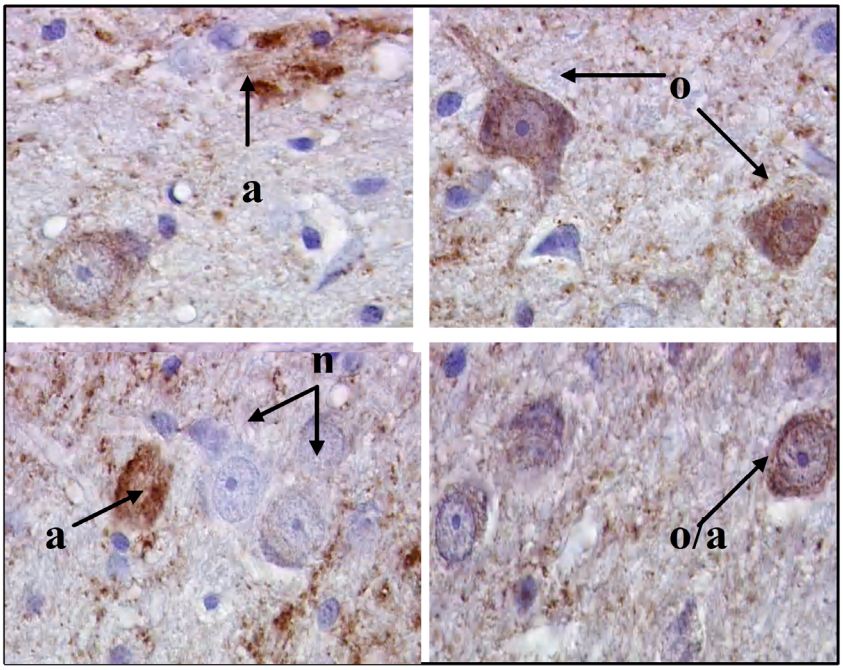


Figure 5: *Intracerebral rotenone model: Examples of possible α-synuclein overexpression (**o**) and aggregation (**a**) in dopaminergic cells compared to non-expressing cells (**n**) within the SNc of rats with rotenone-induced lesions of the MFB. Images are at 100x magnification, α-synuclein was visualised with DAB and a haematoxylin counterstain.*

Intracerebral model

The histological evaluation of the intracerebral infusion of rotenone indicated that there was no effect of the aECF infusion on the percentage of striatal TH immunostaining (Table 3 and Figure 3A), while there was a dose-dependent decrease in TH staining in rats with rotenone infusion. The dose-dependent nature of the toxin effect was most pronounced on day 14 after infusion. However, while the damage appeared to be progressive, there were no significant differences among any of the rotenone doses on day 28 compared to day 14 and a time-dependent decrease can only be observed for the 5.0 µg rotenone dose. Also, there appears to

be an increase in TH staining in both the striatum and SNc for the 2.0 µg dose between day 14 and 28. The TH immunostaining of the SNc (Table 4 and Figure 3B) showed that aECF infusion did not alter the number of TH positive nigral cells, but infusion of rotenone produced dose-dependent decrease in cell number, although less pronounced than that observed in the striatum.

The results of α-synuclein immunostaining of the SNc indicated that there were α-synuclein positive cells in a few of the rotenone treated rats (Figure 4 and Figure 5). The α-synuclein staining was variable among rats while there were more positive cells in some of the rotenone treated rats, without an increase α-synuclein staining in several others.

Peripheral organ pathology

Subcutaneous model

Four rats (20%) died spontaneously after 3-5 days of rotenone administration (3.0 mg/kg/day). No peripheral organ pathology was performed on these rats. In the surviving rats, significant treatment-related changes were observed in the stomach and minor changes were observed in the liver and heart. These changes were attributed to rotenone administration and are summarised in Table 5. Some minor changes were also seen in the liver and adrenals. The latter changes were most probably the consequence of decreased food consumption or complete anorexia and stress, respectively.

Table 5: *Subcutaneous rotenone model: Incidence (%) of rotenone-related histopathological changes in Lewis rats (n=56) sacrificed on schedule.*

Scheduled Sacrifices	**Day 14**			**Day 28**			**All Day Combined**		
Groups	saline n=10	vehicle n=10	rotenone n=8	saline n=10	vehicle n=10	rotenone n=8	saline n=20	vehicle n=20	rotenone n=16
Stomach									
Mineralisation, minimal to moderate	0	0	2 (25%)	0	0	5 (63%)	0	0	7 (44%)
Forestomach mucosal thinning, min to slight	0	0	2 (25%)	0	0	1 (13%)	0	0	3 (19%)
Heart									
Myocardial Necrosis, minimal	0	0	2 (25%)	0	0	0	0	0	2 (13%)
Liver									
Decreased glycogen content, marked to severe	0	0	7 (88%)	0	0	5 (63%)	0	0	12 (75%)
Centrilobular Single Cell Necrosis, slight	0	0	2 (25%)	0	0	0	0	0	2 (13%)
Increased Centrilobular Inflammation, slight	0	0	1 (13%)	0	0	0	0	0	1 (6%)
Oil Red O Negative Microvacuolation, slight	0	0	1 (13%)	0	0	0	0	0	1 (6%)
Adrenal									
Mineralisation, cortex, minimal, unilateral	0	0	1 (13%)	0	0	0	0	0	1 (6%)
Increased cortical vacuolation, min to slight	0	0	2 (25%)	0	0	0	0	0	2 (13%)

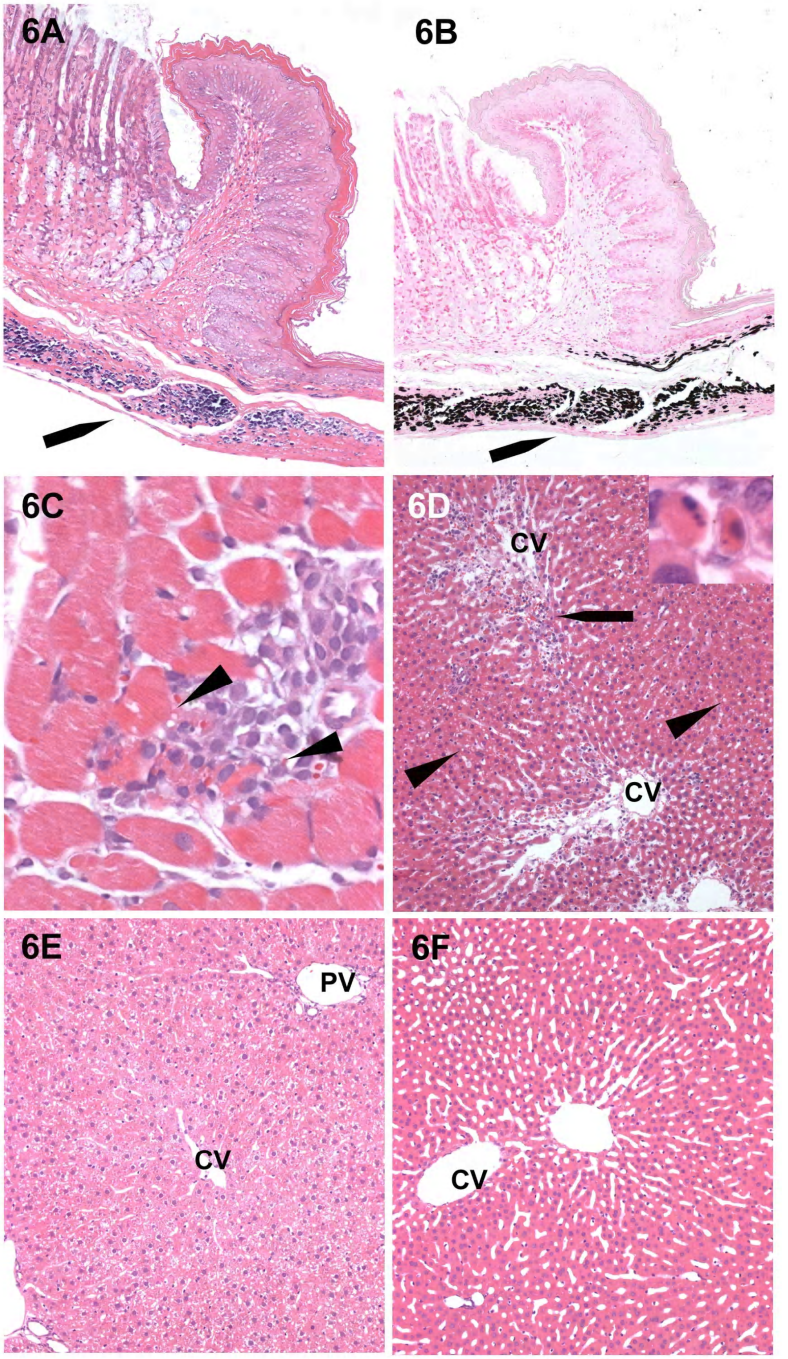
Time related increase in incidence and severity of gastric mineralisation, confirmed by a Von Kossa stain, was observed in the smooth muscles fibers, the elastic connective tissue and occasionally in the glandular epithelial cells of the muscular layer, the submucosa, the muscularis mucosae and glandular crypts of the stomach (Figure 6A and Figure 6B). In the heart, minimal myofiber necrosis characterised by significant coagulative necrosis of myofibers and an inflammatory response was observed in two rotenone treated rats sacrificed on day 14 (Figure 6C). In the liver, slight increase in centrilobular single cell necrosis and/or inflammation was noted in 2 rats sacrificed on day 14, above what is usually seen in overnight fasted albino rat controls (Figure 6D). A decrease in

glycogen content was found in a majority of rats (Table 5), which was characterised by a marked decrease or an absence of clear cytoplasmic spaces without sharp outlines, a smaller size of hepatocellular trabeculae and was accompanied by centrilobular hepatocellular atrophy in the most severe cases (Figures 6E-F). Even though it is only of minor importance since it is anorexia-related, the change was clearly the most dramatic in terms of incidence and severity. In the adrenal, minimal multifocal mineralisation (calcification) was seen in the cortex of a single rat sacrificed on day 28.

Intracerebral model

For the intracerebral rotenone model, no dose or time related changes were observed in the lungs, heart, liver, stomach, spleen, kidney or adrenals of rats given up to 5.0 µg of rotenone up to 28 days, suggesting no peripheral toxicity.

Figure 6: *Subcutaneous rotenone model:* ***6A:*** *Limiting ridge of the stomach, Lewis rat given 3.0 µg/day of rotenone for 28 days. The muscularis and submucosa have extensive mineralisation (arrow). Hematoxylin and Eosin (HE) stain. Original magnification 100x.* ***6B:*** *Serial section of the change observed in figure 6A stained according to von Kossa's method for calcium precipitates (arrow). Original magnification 100x.* ***6C:*** *Right ventricular free wall of the myocardium, Lewis rat given 3.0 µg/day of rotenone for 14 days. Minimal focus of myocardial necrosis (arrowheads) and adjacent perivascular mononuclear cell infiltration. HE. Original magnification 400x.* ***6D:*** *Median lobe of the liver of a Lewis rat given 3.0 µg/day of rotenone for 14 days. Slightly increased single cell necrosis (inset) and slight inflammation (aggregates of mixed cells - arrow) are observed in numerous centrilobular areas adjacent to the central veins (CV), along with slight diffuse cytoplasmic hepatocellular vacuolation (arrowheads). Occasional focal widening of the perivenous centrilobular spaces suggests loss of centrilobular hepatocytes. HE. Original magnification 100x.* ***6E:*** *Median liver lobe from a Lewis rat given saline for 14 days. Clear cells with empty spaces (interpreted as washed-off glycogen storage) and a few vacuolation (interpreted as lipid droplets) typical of normal liver of a non-fasted rat. CV: central vein. PV: portal vein. HE. Original magnification 100x.* ***6F:*** *Median lobe of the liver of a Lewis rat given 3.0 µg/day of rotenone for 28 days. Simple severe thinning of hepatic cords due to hepatocellular atrophy, with loss of clear cytoplasm and lipid vacuoles, interpreted as loss of glycogen content, typical of rats with anorexia. HE. Original magnification 100x.*


6A
6B
6C
6D
CV
CV
6E
PV
CV
6F
CV

Microdialysis experiment

Subcutaneous model

For the subcutaneous rotenone model, all microdialysis concentrations were corrected for in vitro recovery (10.6 ± 0.7 %) in order to estimate brain extracellular ($brain_{ECF}$) concentrations. No statistically significant difference was found in concentration-time profiles or in area under the curves (AUC) in plasma among any of the groups indicating that the treatment with rotenone or the vehicle had no effect on the overall disposition of fluorescein (Figure 7A and Table 6). However a significant difference in the estimated $brain_{ECF}$ profiles was seen between rotenone treated rats and saline treated rats ($P<0.05$) and between vehicle-treated rats and saline-treated rats ($P<0.01$) from 80-150 minutes (Figure 8A). The BBB transport of fluorescein was calculated as the ratio of AUC_{ECF} and AUC_{plasma} multiplyed by 100%. The results are shown in Table 6. No statistically significant difference was seen among all treatment groups.

	Subcutaneous rotenone model		
	AUC_{plasma} (µg/ml*min) Mean ± SEM	**AUC_{ECF}** (µg/ml*min) Mean ± SEM	**AUC ratio** (µg/ml*min) Mean ± SEM
Saline	1007 ± 200	3.0 ± 0.4	0.30 ± 0.20
Vehicle	1029 ± 117	7.1 ± 1.6	0.69 ± 1.40
Rotenone	1502 ± 306	18 ± 5	1.22 ± 1.64
	Intracerebral rotenone model		
	AUC_{plasma} (µg/ml*min) Mean ± SEM (%)	**AUC_{ECF}** (µg/ml*min) Mean ± SEM (%)	**AUC ratio** (µg/ml*min) Mean ± SEM (%)
aECF (control)	642 ± 42	1.6 ± 0.2	0.25 ± 0.03
aECF (infused)		2.3 ± 0.7	0.36 ± 0.10
Rotenone (control)	642 ± 42	1.6 ± 0.2	0.25 ± 0.03
Rotenone (infused)		2.6 ± 1.2	0.40 ± 0.23

Table 6: *Mean area under the curves (AUC in µg/ml*min) of the plasma and estimated extracellular fluid (ECF) curves for both the subcutaneous rotenone model as well as the intracerebral rotenone model as shown in Figure 7 and Figure 8. To determine transport of fluorescein across the BBB, the AUC_{ECF}/AUC_{plasma} ratio was calculated. The data are presented as mean values ± SEM.*

Intracerebral model

For the intracerebral rotenone model, the concentration-time profiles of fluorescein in plasma are shown in Figure 7B. All microdialysate concentrations were corrected for the average *in vivo* recovery as determined during the retrodialysis period (25 ± 1 %) in order to estimate $brain_{ECF}$ concentrations. The *in vivo* recovery was equal for the two concentrations of fluorescein used. The concentration-time profiles of fluorescein in $brain_{ECF}$ are shown in Figure 8. No significant differences were found in area under the curves (AUC) in plasma or in $brain_{ECF}$ among any of the groups indicating that the treatment with rotenone or DMSO/PEG had no effect on the overall disposition of fluorescein (Table 6). No statistical difference was observed in overall BBB transport of fluorescein among any of the groups as indicated by the AUC ratios (Table 6).

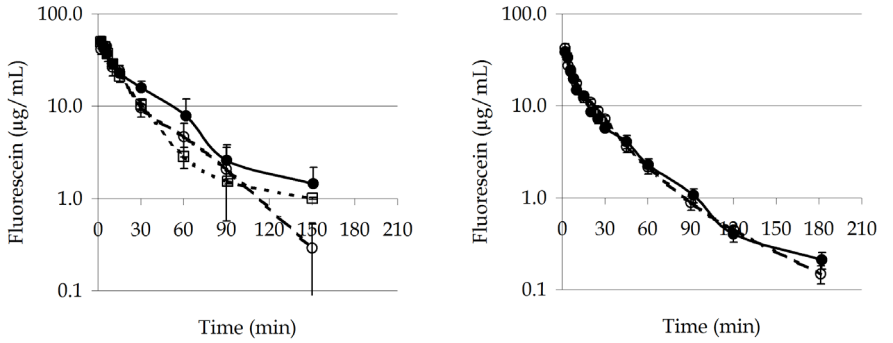


Figure 7: (Left panel) *Plasma fluorescein concentration (µg/mL) vs. time (min) on day 14 after the subcutaneous infusion of* (o) *saline,* (□) *vehicle (DMSO/PEG) or* (•) *3.0 mg/kg/day rotenone. The data are expressed as mean values ±SEM.* ***(Right panel)*** *Plasma fluorescein concentration (µg/mL) vs. time (min) on day 14 after an intracerebral infusion of* (o) *artificial ECF or* (•) *5.0 µg rotenone. The data are mean values±SEM. Statistical analysis (one-way ANOVA) showed no significant difference between all groups for both rotenone models.*

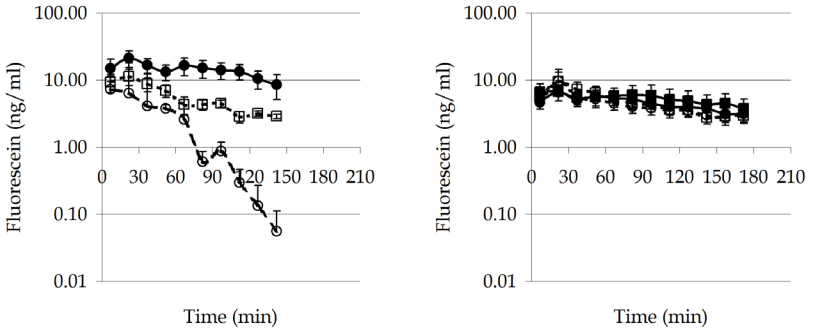


Figure 8: (Left panel) *Microdialysate fluorescein concentration (µg/mL) vs. time (min) on day 14 after the subcutaneous infusion of (o) saline, (□) vehicle (DMSO/PEG) or (•) 3.0 mg/kg/day rotenone. A significant difference was seen (time>60 min) between rotenone- and saline treated rats (P<0.05; Student's unpaired t-test) and between vehicle- saline treated rats (P<0.01; Student's unpaired t-test).*

(Right panel) *Microdialysate fluorescein concentration (µg/mL) vs. time (min) on day 14 after an intracerebral infusion of (o, □) artificial ECF or (•, ■) 5.0 µg rotenone. Circles represent the control-side of the brain and squares represent the infused side of the brain.The data are mean values±SEM. Statistical analysis (one-way ANOVA) showed no significant difference between all groups.*

4. Discussion

In this study we compared the subcutaneous and intracerebral administration of rotenone to develop a rat model for Parkinson's disease. We examined brain histology, peripheral toxicity, behaviour and BBB permeability.

Subcutaneous model

The subcutaneous infusion of rotenone resulted in minor development of dopaminergic lesions whereas profound peripheral organ toxicity was found. Although the subcutaneous infusion seemed to affect locomotor behaviour and seemed to induce an increase in BBB permeability compared to the control infusion (saline and vehicle), these results could not be correlated to nigrostriatial injury, but were most probably a consequence of peripheral organ toxicity. A time-related increase in incidence and severity of mineralisation (calcification) in the stomach was observed in rotenone-treated rats and is clearly related to the

systemic rotenone administration. Since there were no significant kidney lesions, the mineralisation is probably dystrophic secondary to cellular injury/necrosis, instead of metastatic associated with hypercalcemia or systemic Ca/P imbalance related to renal failure (uremia) (Cotran *et al.*, 1999; Palmer, 1993). However, this change would deserve further investigations to definitively rule out a metastatic mechanism. The mineralisation (calcification) in the stomach of rotenone-treated rats could be the consequence of mitochondrial injury due to inhibition of complex I and/or oxidative stress and secondary calcium influx (Sousa et al., 2003; Sousa and Castilho, 2005). The reason as to why the damaged is localised to the stomach remains unsolved. A reduced blood-flow to the stomach as a result of chronic excessive stomach distension could be an explanation, although mucosal thinning which is indicative of excessive distension was not consistently observed. This change has not been, to our knowledge, previously reported in rats that received rotenone systemically. Stomach distension is usually a non-specific change related to physiological demise, nausea (pica), severe stress or exaggerated pharmacological effect and there were no morphological evidences of atrophy of parietal cells, which could support the previously published hypothesis of achloridria with decreased wall mass (Lapointe *et al.*, 2004). The observed cardiac changes in 2 rotenone treated rats were attributed to treatment, since none were observed in any of the saline or vehicle treated rats and this change is not common in albino rats of that age; however, a background change cannot strictly be ruled out, since this change may occasionally be found in control Fischer 344 or Sprague Dawley rats (Boorman *et al.*, 1990). Liver changes were minor and related to decreased bodyweight with time and suspected decrease in food consumption. All of these changes could be secondary to the gastric lesions, especially the observed decrease in glycogen content. In addition, a few animals had atrophy of hepatocellular cords along with increased single cell necrosis, inflammation and hepatocellular microvacuolation. Although these changes could be associated with starvation, a rotenone-related effect cannot be ruled out. In contrast to a previous report with similar experimental conditions, hepatocellular coagulative necrosis was not reported in this study (Lapointe *et al.*, 2004).

The peripheral toxicity, high mortality and variability in the development of dopaminergic lesions in the brain of systemically administered rotenone as we have seen in our experiments is confirmed by literature (Betarbet *et al.*, 2000; Fleming *et al.*, 2004; Hoglinger *et al.*, 2003; Lapointe *et al.*, 2004; Sherer *et al.*, 2003; Zhu *et al.*, 2004) although it has not been so extensively investigated as in the

experiments described in this paper. In conclusion, these results indicate that the subcutaneous infusion of rotenone in the rat causes significant systemic toxicity, notably important and adverse changes in the stomach and heart, while not able to produce a sufficient amount of nigrostriatal damage as referred to as golden standard for Parkinson's disease.

Intracerebral model

The intracerebral infusion caused a progressive, dose-dependent decrease in dopaminergic neurons to about 30% in 28 days, with in a few cases α-synuclein immunoreactivity and aggregation but without any peripheral toxicity or any effect on bodyweight. Although the α-synuclein inclusions were not directly related to the severity of the dopamine lesions, they have not been observed in previous experiments using an intracerebral infusion of rotenone (Alam *et al.*, 2004; Antkiewicz-Michaluk *et al.*, 2004; Saravanan *et al.*, 2005; Sindhu *et al.*, 2005) and this result is therefore one step closer to mimicking the human pathology of Parkinson's disease. Saravan et al observed a dose-depentdent (2, 6 and 12 μg) decrease was in striatal biogenic levels already on day 5 after the infusion (Saravanan *et al.*, 2005). In our studies, we used a lower concentration range which may account for the fact that we do not see a clear dose-dependent decrease in the first 4 to 7 days. Furthermore, the decrease TH staining in time is most evident at 5.0 μg, specifically in the SNc. In the striatum the decrease seems to reach a minimal plateau after 28 days. Moreover, there appears to be an increase in TH staining in both the striatum and SNc for the 2.0 μg dose between day 14 and 28. This could perhaps be explained by the relatively low concentration infused, some rats being less susceptible to rotenone and general variability wit this toxin. Variability has also been observed in response to systemically treated rotenone rats (Betarbet *et al.*, 2000; Sherer *et al.*, 2003). It would be of interest to look at higher doses and for a longer period of time and perhaps compare different strains of rats specifically for the infusion of rotenone into the MFB. The amphetamine induced rotational behaviour of the intracerebral infused rotenone rats (5.0 μg) showed a significant preference of these rats for rotating in the ipsiversive direction on day 21 which was consistent with a unilateral lesion in the brain dopaminergic system. These results were somewhat different from a study by Sindhu *et al.* (2005) where they did not see any significant stereotypic rotations in MFB-lesioned rats (12 μg) until day 28. Again these difference may be due to variation among rat strains (Lewis rats compared to Sprague-Dawley rats).

In contrast to what would be expected on the basis of literature (Alexander *et al.*,

1994; Carvey *et al.*, 2005; Kortekaas *et al.*, 2005), no difference in BBB permeability was found in the striatum in the Parkinson's diseased brain at 14 days post-injection. It remains to be answered if changes would occur at other post-injection intervals or at other locations in the brain because microdialysis (being a good technique to study BBB transport characteristics as it monitors in time (de Lange *et al.*, 1997; Hammarlund-Udenaes *et al.*, 1997)) does not provide spatial information. In our view further investigations on BBB transport mechanisms are needed, to be performed in a strict mechanistic and quantitative manner, as a function of the disease-state.

The intracerebral administration of rotenone creates the opportunity to develop an animal model for Parkinson's disease, in which the toxin is delivered in its target brain area thereby reducing side-effects in other parts of the body that may interfere in the investigations, with features getting closer to those in Parkinson's disease patients. However, upon local delivery of the toxin, the question remains to be answered on how toxins which are capable of inducing Parkinson's disease in human are able to reach their target in the brain. Further experiments to follow the progression at longer post-injection intervals would be required to evaluate if indeed there is further disease progression and further α-synuclein development beyond 28 days. Since age seems to increase the sensitivity of dopaminergic neurons to rotenone toxicity (Phinney *et al.*, 2006), it would be worthwhile to investigate this for the IC infused rotenone rats and the influence that age might have on the development of α-synuclein inclusions.

Comparing these results to the more commonly used 6-OHDA model, in our hands, 6-OHDA infusion into the nigral cell bodies (O'Neill *et al.*, 2004) or into the MFB (Visanji *et al.*, 2006) produces a more rapid (5-10 days) loss in terminals. Also, when infused into the SNc, 6-OHDA causes an anterograde degeneration of the whole nigrostriatal dopaminergic system (O'Neill *et al.*, 2004; Sachs and Jonsson, 1975), resulting in a more rapid loss of TH positive cells. Thus, the seemingly slower effects of MFB infused rotenone obtained thusfar favours a more chronic model than 6-OHDA. This might be due to differences in the solubility, stability, and diffusion of the toxin or to the mechanism by which the toxin produces cell death. 6-OHDA can be infused into the striatum and while a rapid loss in dopamine terminals is observed, this model also produces slow progressive retrograde damage to the nigral cells (Murray *et al.*, 2003; Rosenblad *et al.*, 1999), however, so far no α-synuclein inclusions have been observed in this model. In conclusion, the data presented here indicate that rotenone infused intracerebrally is able to create a progressive rat model for Parkinson's disease.

5. Reference List

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Chapter 5

The Intracerebral Rotenone Model of Parkinson's Disease in Rats:

Altered Conversion of L-DOPA into DOPAC and HVA without Changes in BBB Transport

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Abstract

The aim was to study the pharmacokinetics (PK) and blood-brain barrier (BBB) transport in conjunction with the conversion of L-DOPA into DOPAC and HVA in the rat rotenone model of Parkinson's disease.

Male Lewis rats were unilaterally infused with rotenone in the medial forebrain bundle (MFB). The contralateral side served as a control. After 14 days, L-DOPA (10, 25 or 50 mg/kg) was intravenously infused. Blood samples and brain striatal microdialysates were collected and analysed. Brains were histologically evaluated on tyrosine hydroxylase staining to identify responders and non-responders. Population PK analysis was performed by NONMEM to determine the influence of the Parkinson's disease state on plasma PK, BBB transport of L-DOPA and DOPAC and HVA levels in the brain.

Rotenone infusion resulted in 71% of the rats developing neurostriatal degeneration. The plasma PK of L-DOPA could be adequately described by a 3-compartmental model. No difference was observed between BBB transport of L-DOPA in lesioned and untreated brain side. DOPAC and HVA basal microdialysate levels were substantially lower in the lesioned striatum, while also, following L-DOPA administration, their elimination rates were higher in lesioned striatum.

Our study demonstrates that Parkinson's disease-like pathology induced by rotenone does not result in changes in the kinetics and BBB transport of L-DOPA. Merely, a clear effect of disease on the levels and elimination rates of DOPAC and HVA in brain were found, providing information on decreased dopamine concentrations at the lesioned brain side.

1. Introduction

Parkinson's disease is a progressive neurodegenerative disease in which dopamine-producing cells in the nigrostriatal pathway are affected. The chronic and progressive decrease in dopamine concentration in the striatum leads to the development of the classic symptoms of Parkinson's disease, such as tremor, rigidity and bradykinesia (Dauer and Przedborski, 2003). The current therapies of Parkinson's disease are mainly focussed on symptomatic treatment by replacing the striatal dopamine (Factor, 2008; Nyholm, 2006; Savitt *at al.*, 2006; Schapira, 2008). Among the antiparkinson drugs available, L-3,4-dihydroxyphenylalanine (L-DOPA) is still considered the most efficacious treatment of Parkinson's symptoms (Deleu *at al.*, 2002; Factor, 2008; Nyholm, 2006; Schapira, 2008).

To investigate the mechanisms of action of antiparkinsonian drugs, one would like to use a preclinical Parkinson's disease model that allows for investigation at different stages of the disease. In **chapter 4**, we have explored the intracerebral rotenone rat model for Parkinson's disease, which showed to be capable of producing a relatively slow progressive degeneration of the nigrostriatal system and was associated with the formation of inclusion bodies similar to that observed in Parkinson's disease (Ravenstijn *at al.*, 2008).

The goal of this study was to determine the PK in plasma, BBB transport, and within-brain conversion of L-DOPA into dopamine and its major metabolites DOPAC and HVA in this rat model for Parkinson's disease. The microdialysis technique (de Lange *at al.*, 1999; Hammarlund-Udenaes, 2000) was used to measure free L-DOPA, dopamine, DOPAC and HVA concentrations. To examine the influence of Parkinson's disease, two microdialysis probes were inserted in the lesioned side and in the contralateral healthy side of the brain respectively. Microdialysates were collected in parallel with serial blood samples. Concentrations of the compounds were determined under basal conditions and following *i.v.* administration of 10, 25 and 50 mg/kg of L-DOPA. At the end of the experiment brains were removed for immunostaining on tyrosine hydroxylase (TH) to determine responders and non-responders on the basis of the percentage loss of dopaminergic terminals in the striatum in control and rotenone-infused rats. A compartmental, population-based analysis was performed to determine the influence of the Parkinson's disease state on plasma PK, BBB transport of L-DOPA and DOPAC and HVA levels in the brain.

2. Materials and Methods

All animal procedures described in this paper were approved by the Ethical Committee on Animal Experimentation of the University of Leiden (DEC numbers 118 and 5069).

Animals and surgical procedures

Experiments were performed on male Lewis rats (Charles River BV, Maastricht, The Netherlands) weighing 311 ± 17g (mean ± s.d., n=18) and 288 ± 13g (mean ± s.d., n=17) before surgery and before start of the experiment, respectively. The rats were housed in standard plastic cages (six per cage before surgery and individually after surgery) with a 12-hour day/night schedule (lights on 7:30 AM) and at a temperature of 21°C.

The animals had access to standard laboratory chow (RMH-TM; Hope Farms, Woerden, The Netherlands) and acidified water *ad libitum*.
The surgery was performed under anesthesia with an intramuscular injection of 0.1 mg/kg medetomidine hydrochloride (Domitor 1 mg/ml, Pfizer, Capelle a/d IJssel, The Netherlands) and 1 mg/kg ketamine base (Ketalar 50 mg/ml, Parke-Davis, Hoofddorp, The Netherlands). Three indwelling cannulas (pyrogen-free, nonsterile polyethylene tubing, Portex Limited) were implanted, one in the left femoral artery (for blood sampling) and two in the left femoral vein (for drug administration). The cannulas were tunnelled subcutaneously and fixed at the back of the neck with a rubber ring. The skin in the neck was stitched with normal sutures. The skin in the groin was closed with wound clips. To prevent clotting and cannula obstruction, the cannulas were filled with a 25% (w/v) polyvinylpyrrolidone solution (PVP; Brocacef, Maarssen, The Netherlands) in pyrogen-free physiological saline (B. Braun Melsungen AG, Melsungen, Germany) containing 20 IU/ml heparin (Hospital Pharmacy, Leiden University Medical Center, Leiden, The Netherlands).

After the implantation of the blood cannulas, the rats were placed in a stereotaxic frame and the skull was exposed for brain surgery. The skull was cleaned and a hole was burred to allow a needle to be lowered into the right median forebrain bundle (MFB: AP: -2.8; L: +2.0; V: -9.0 relative to bregma (Paxinos *at al.*, 1985)) for unilateral infusion of 5.0 µg of rotenone (Rotenone Pestanal ; Sigma Alldrich BV, Zwijndrecht, the Netherlands) at a rate of 0.1 µl/min for 30 minutes. Rotenone was dissolved in a 1:1-mixture of dimethylsulfoxide (DMSO, Sigma Alldrich BV, Zwijndrecht, the Netherlands) with polyethylene glycol (PEG 200, Sigma Alldrich BV, Zwijndrecht, the Netherlands). After the infusion, the needle was kept in place for another 5 minutes to allow diffusion of the fluid without leakage along the track of the needle. Subsequently, two small holes were drilled into the skull to allow implantation of a microdialysis guide cannula (CMA/12, Aurora Borealis Control B.V. Schoonebeek, The Netherlands) in the left and in the right striatum (AP: +0.4; L: +/-3.2; V: -3.5, relative to bregma; (Paxinos *at al.*, 1985)). One support screw was placed as an extra anchor for fixation of the guide, which was glued to the skull with dental acrylic cement (Howmedia simplex rapid + methylacrylate, Drijfhout, Amsterdam, The Netherlands). After surgery, the rats were assigned to one of three dosing groups (n=6 per group). The microdialysis experiment was carried out 14 days after surgery. The details of the intracerebral rotenone model have been described in **Chapter 4**.

Microdialysis

At 13 days after the unilateral infusion of rotenone into the right MFB, and 18-24 hours prior to the experiment, the microdialysis probes (CMA12, membrane length of 4.0 mm; Aurora Borealis Control B.V. Schoonebeek, the Netherlands) were inserted into the guide cannulas. All animals were fasted overnight prior to the experiment in order to rule out any competition in BBB transport of L-DOPA with food-related amino acids (Nutt *at al.*, 1984).

The microdialysis experiment, 14 days post rotenone treatment, was started between 7:00 and 8:00 a.m. The inlets of the microdialysis probes were connected by FEP tubing (fluorinated ethylene propylene tubing; Aurora Borealis Control B.V. Schoonebeek, the Netherlands) to syringe pumps (Beehive, Bas Technicol, Congleton, United Kingdom). The probes were perfused with artificial ECF (composition in mM: NaCl 145; KCl 2.7; CaCl2 1.2; MgCl2 1.0; ascorbic acid 0.2 in a 2 mM phosphate buffer pH 7.4; (Moghaddam and Bunney, 1989)) at a flow rate of 2 µl/min. The outlets also consisted of FEP tubing and were connected to a microsample collector (Univentor 820; Antec, Leiden, The Netherlands), in which the samples were collected at 4^{o}C. The vials contained an antioxidant fluid (0.1 M acetic acid, 3.3 mM L-cysteine, 0.27 M EDTA, 0.0125 mM ascorbic acid dissolved in millipore water) in a ratio of 1:4 with the expected volume of the microdialysate to prevent the breakdown of the catecholamines. After a stabilisation period of 60 minutes, the *in vivo* recovery of L-DOPA was determined by the retrodialysis method. For this purpose, the probes were perfused with a L-DOPA solution (10, 100 or 200 ng/ml for the 10, 25 and 50 mg/kg dose group, respectively) for 60 minutes to collect 6 fractions. The relative loss of L-DOPA was used for estimating $brain_{ECF}$ concentrations. After the retrodialysis, the syringes were switched to blank perfusion fluid for a washout phase of 90 minutes. After the washout period, the intravenous administration of L-DOPA was started. One venous cannula was connected to a syringe containing L-DOPA (Sigma Alldrich BV, Zwijndrecht, the Netherlands) in 0.2 M HCl in saline (0.9% NaCl) and ascorbic acid (5% of the L-DOPA amount) and the second venous cannula was connected to a syringe containing 7% $NaHCO_3$ (to neutralise the acidic L-DOPA solution). Both infusions were started at the same time for 20 minutes at a rate of 20 µl/min. In the first 120 minutes of the experiment, microdialysis fractions were collected at 10-minute intervals. From 120-180 minutes, microdialysis fractions were collected at 20-minute intervals. From 180 minutes until the end of the experiment (360 minutes), microdialysis fractions were collected at 30-minute intervals. Blood samples (50 µl in heparinised Eppendorf vials) were taken predose and at 5, 10,

15, 20, 22, 24, 26, 28, 30, 45, 50, 60, 75, 90, 120, 180, 240, 360 minutes after start of the L-DOPA infusion. The blood samples were centrifuged for 10 minutes at 5000 rpm and the plasma was pipetted into Eppendorf vials. All samples were stored at -80 C before analysis. After the experiment, the animals were given an overdose of sodium pentobarbital (Nembutal, Ceva Santa Animale, Maassluis, The Netherlands) and the thorax was opened and the vascular bed was perfused via the left ventricle of the heart with 30 ml of saline followed by 30 ml of 10% phosphate buffered formalin (pH 7.0). Brains were removed for histopathology.

Immunohistopathology

TH immunohistochemistry was performed to quantify the degree of dopaminergic depletion as described previously in **Chapter 4**. The percentage of intact TH staining in the rotenone-treated hemisphere was calculated as the percentage of striatal MGV compared to the striatal MGV of the untreated hemisphere. In the clinical setting, symptoms of Parkinson's disease arise when about 80% of striatal dopamine and about 60% of dopamine neurons are lost (Dauer and Przedborski, 2003). Therefore, the rats which failed to show significant loss in TH staining were considered as 'non-responders' to the rotenone treatment.

Bioanalysis of L-DOPA, DOPAC and HVA

All plasma samples were analysed for L-DOPA and all microdialysate samples were analysed for L-DOPA, dopamine, DOPAC and HVA using a high performance liquid chromatography (HPLC) system with electrochemical detection (ECD).

HPLC and ECD system

The HPLC system consisted of a LC-10AD HPLC pump (Shimadzu, 's Hertogenbosch, The Netherlands), a Waters 717 Plus autosampler (Waters, Etten-Leur, The Netherlands), a pulse damper (Antec Leyden, Zoeterwoude, The Netherlands) and a digital electrochemical amperometric detector (DECADE, software version 3.02, Antec Leyden, Zoeterwoude, The Netherlands). The electrochemical detector consisted of a VT-03 electrochemical flow cell combined with a 25 µm spacer and an *in situ* Ag/AgCl (ISAAC) reference electrode operating in the DC mode. For the analysis, a standard Ag/AgCl reference electrode, filled with a saturated KCl solution was used. Data acquisition and processing was performed using the Empower® data-acquisition software (Waters, Etten-Leur, The Netherlands).

Bioanalysis in plasma

Chromatography of plasma samples of L-DOPA was performed on a Beckman Coulter™ Ultrasphere® 5 µm C-18 column (4.6 mm I.D. x 150 mm, Alltech, Breda, The Netherlands) equipped with a refill guard column (2 mm I.D. x 20 mm, Upchurch Scientific, Oak Harbor, WA, USA) packed with pellicular C18 material (particle size 20-40 µm, Alltech, Breda, The Netherlands) at a constant temperature of 30°C. The mobile phase was a mixture of 0.05 M sodium phosphate buffer (pH 2.8) and methanol (90:10, v/v), supplemented with 0.3 mM EDTA (sodium salt) and 10 mM octane-sulfonic acid. Before the addition of methanol, the mobile phase was filtered through a 0.2 µm nylon filter (Alltech, Breda, The Netherlands), then the methanol was added and it was mixed and degassed with helium. The flow rate was set at 1 mL/min. The optimal working potential for L-DOPA was +0.75 V, as determined by a voltammogram and sensitivity plot. Concentrations were measured at a sensitivity range of 5 nA for L-DOPA and 20 nA for 3,4-dihydroxybenzylamine hydrobromide (DHBA; internal standard). Stock solutions of L-DOPA were prepared at a concentration of 1 mg/mL in Millipore water. The stock solutions were diluted with Millipore water to obtain calibration solutions in the range of 2 to100 ng/mL. The internal standard (DHBA) solution was prepared by dilution of the stock solution to a final concentration of 500 ng/mL. The stock solutions were stored at -80 °C up to one month. The assay solutions were prepared freshly before each analysis. For determination of the L-DOPA in plasma, 25 µL of internal standard solution (DHBA 500 ng/mL) was added to 45 µL plasma samples and 50 µL of Millipore water in glass centrifuge tubes. Next, 25 µL of 20% TCA was added and the mixture was vortexed for 5 min. After centrifugation for 10 min at 4000 rpm (2000g), 100 µL of the supernatant was added to 50 µL of phosphoric buffer (1M, pH 5.5) of which 25 µL was injected into the HPLC system.

Bioanalysis in microdialysate

For analysis of L-DOPA, dopamine, DOPAC and HVA brain microdialysate concentrations, 5 µL of internal standard (isoproterenol; 100 ng/mL) solution was added per 10 µL of microdialysis sample or calibration curve sample. The samples were then injected (20 µL) into the HPLC system without further sample pre-treatment. Chromatography of brain microdialysate samples was performed on a Beckman Coulter™ Ultrasphere® 5 µm C-18 column (2 mm I.D. x 250 mm, Alltech, Breda, The Netherlands) at a constant temperature of 30 °C. The mobile

phase was a mixture of 0.05 M sodium phosphate buffer (pH 2.8) and methanol (88:12, v/v), supplemented with 0.3 mM EDTA (sodium salt) and 1.5 mM octane-sulfonic acid. Mobile phase solvents were filtered through a 0.2 µm nylon filter. Then, the methanol was added and the mobile phase was mixed and degassed with helium. The flow rate was set at 0.2 mL/min. The optimal working potential for a mixture of L-DOPA, dopamine, DOPAC and HVA was +0.66 V, as determined by a voltammogram and sensitivity plot. Concentrations were measured at a sensitivity range of 0.1 nA for dopamine, 0.5 nA for L-DOPA, HVA and isoproterenol; and 10 nA for DOPAC. For analysis of brain microdialysate samples, stock solutions of L-DOPA, DOPAC and HVA were prepared at a concentration of 0.5 µg/mL for dopamine, 1 µg/mL for L-DOPA and 5 µg/mL for DOPAC and HVA in microdialysis perfusion fluid with aqueous antioxidant solution consisting of 0.1 M acetic acid, 3.3 mM L-cysteine, 0.27 M EDTA (sodium salt) and 0.0125 mM ascorbic acid (4:1 v/v). Internal standard solution was prepared fresh before each analysis by dilution of a 1 mg/mL isoproterenol stock solution to 100 ng/mL perfusion fluid with antioxidant (4:1 v/v). All the stock solutions were stored at -80 °C up to one month. Before each analysis a first calibration solution containing all compounds was freshly prepared by mixing one volume part of each compounds' stock solution (L-DOPA, dopamine, DOPAC and HVA) and adding 5 volume parts of perfusion fluid with antioxidant (4:1 v/v). This first calibration solution now contained all compounds at a concentration 10 times lower than their stock solution and from this solution the other calibration solutions were prepared.

Population pharmacokinetic analysis

The pharmacokinetics of L-DOPA, DOPAC and HVA were analysed utilizing a population PK modelling approach. Dopamine concentrations were all below the limit of detection. Compartmental modelling was performed using the ADVAN6 subroutine in NONMEM VI release 2 (GloboMax LLC, Hanover, MD, USA). All fitting procedures were performed on an IBM-compatible computer (Pentium IV, 1500 MHz) running under Windows XP with the Compaq Visual Fortran compiler version 6.6.

The inter-individual variability of model parameters was described by an exponential equation, according to:

$$P_{1i} = \theta_1 * exp(\eta_i),$$

where θ_1 is the population (typical) estimate for parameter P_1, P_{1i} is the individual estimate and η_i determines the random deviation of P_{1i} from P_1. The values of η_i are assumed to be randomly, normally distributed with mean zero and variance ω_{11}^2. The residual error in the L-DOPA concentration was described by a proportional error model:

$$C_{obs,ij} = C_{pred,ij} * (1+\varepsilon_{ij}),$$

and the residual error in the DOPAC or HVA concentration was described by an additive error model:

$$C_{obs,ij} = C_{pred,ij} + \varepsilon_{ij}$$

where $C_{obs,ij}$ represents the j^{th} measured L-DOPA, DOPAC or HVA concentration for the i^{th} individual predicted by the model. $C_{pred,ij}$ represents the prediction of concentration and ε_{ij} is the deviation of the model-predicted value from the observed concentration. The values of ε are assumed to be randomly, normally distributed with mean zero and variance σ^2. The first order conditional estimation method with interaction (FOCE interaction) was used in NONMEM to fit the models to the data and to estimate θ's, ω^2's and σ^2's.

Structural model selection for all models was based on the likelihood ratio test, diagnostic plots (observed concentrations vs. individual and population predicted concentrations, conditional weighted residuals vs. time and predicted concentrations), parameter correlations and precision in parameter estimates. Inclusion of one parameter into the model was assumed to be significant if this led to a decrease of 10.8 points or more of the minimum value of the objective function (MVOF) after fitting the model to the data. This corresponds to a theoretical significance level of p=0.001 under the assumption that the difference in MVOF between two nested models is χ^2 distributed.

In total, the L-DOPA plasma profiles of 13 rats (10 mg/kg: n=4; 25 mg/kg: n=4; 50 mg/kg: n=5) and the L-DOPA $brain_{ECF}$ profiles from the untreated brain side of 12 rats (10 mg/kg: n=4; 25 mg/kg: n=3; 50 mg/kg: n=5) and the L-DOPA $brain_{ECF}$ profiles from the lesioned brain side of 7 rats (10 mg/kg: n=1; 25 mg/kg: n=2; 50

mg/kg: n=4) were included in the population PK analysis. On the basis of selection criteria, the plasma and brain$_{ECF}$ L-DOPA data from all individual rats were simultaneously analysed (Figure 1). Individual model parameters (*post hoc* estimates) for each animal from this analysis and typical values from this model (Table 1) were then used as input for the subsequent analysis of DOPAC (Figure 1; compartments 6 and 8) and HVA (Figure 1; compartments 7 and 9). In total, the DOPAC and HVA microdialysate concentrations from the healthy/untreated brain side of 12 rats (10 mg/kg: n=4; 25 mg/kg: n=3; 50 mg/kg: n=5) and the DOPAC and HVA microdialysate concentrations from the lesioned brain side of 8 rats (10 mg/kg: n=2; 25 mg/kg: n=2; 50 mg/kg: n=4) were included in the population PK analysis.

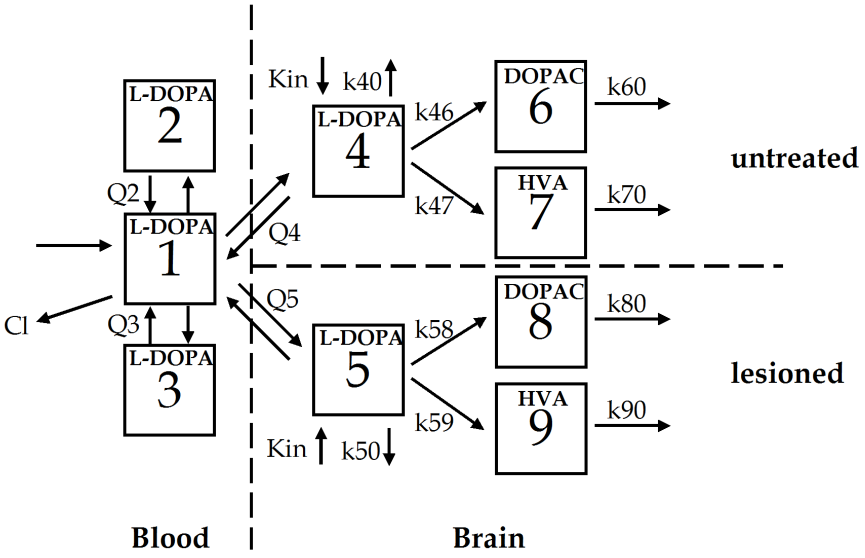

Figure 1: *The population pharmacokinetic model for L-DOPA, DOPAC and HVA comprising of three compartments (1-3) describing the PK of L-DOPA in plasma, two compartments (4 & 5) describing the PK of L-DOPA in brain$_{ECF}$, one for the untreated brain side and one for the lesioned brain side, two compartments (6 & 8) describing the PK of DOPAC in brain$_{ECF}$, one for the untreated brain side and one for the lesioned brain side and two compartments (7 & 9) describing the PK of HVA in brain$_{ECF}$, one for the untreated brain side and one for the lesioned brain side.*

3. Results

General

The percentage of intact TH staining in the rotenone-treated hemisphere compared to the untreated hemisphere was below 40% in 12 out of 17 rats, and higher than 90% in the remaining 5 rats. The rats which exhibited a TH staining level lower than 40% (60-100% of the dopamine terminals were lost) were considered as 'responders' to the rotenone treatment and those brain sides were considered as diseased (lesioned).

No quantifiable concentrations of dopamine could be detected throughout the experiment (LOQ of 0.01 ng/mL in a 20 µl microdialysate sample). As a consequence, dopamine concentrations were not included in the data analysis.

A total of 17 rats (n=6 in the 10- and 25 mg/kg dose group and n=5 in the 50 mg/kg dose group) were used in the microdialysis experiments. All microdialysate concentrations of L-DOPA were corrected for the average *in vivo* recovery as determined during the retrodialysis period (30 ± 6 %) in order to estimate brainECF concentrations. The *in vivo* recovery was equal for the three concentrations of L-DOPA used.

Data analysis

L-DOPA pharmacokinetic modelling

The individual plasma concentration-time profiles following intravenous infusion of L-DOPA are shown in Figure 2. The individual $brain_{ECF}$ concentration-time profiles are shown in Figure 3. The L-DOPA in plasma and in $brain_{ECF}$ (both lesioned and untreated brain sides) were analysed simultaneously. All structural parameters of the population PK model for L-DOPA could be adequately estimated (Table 1). The goodness-of-fit plots for L-DOPA are depicted in Figure 4. No dose-dependency was observed in the plasma PK for L-DOPA.

No significant difference could be detected between the inter-compartmental clearances Q4 and Q5, the volumes of distribution V4 and V5 or the elimination rate constants k40 and k50 when these models were fitted to the L-DOPA brain concentration data. Table 2 shows a summary of the MVOF and parameters estimates after the different assumptions (Q4=Q5, V4=V5, k40=k50 or a combination of any of these assumptions). Also, the separate estimation of endogenous brain production rate of L-DOPA (Kin) for compartment 4 and 5 resulted in similar estimated values (5.8 min^{-1}). Striatal microdialysate baseline L-DOPA levels in the lesioned brain sides were insignificantly lower (P-value=0.07; Welch's t-test) than in the untreated brain side and averaged

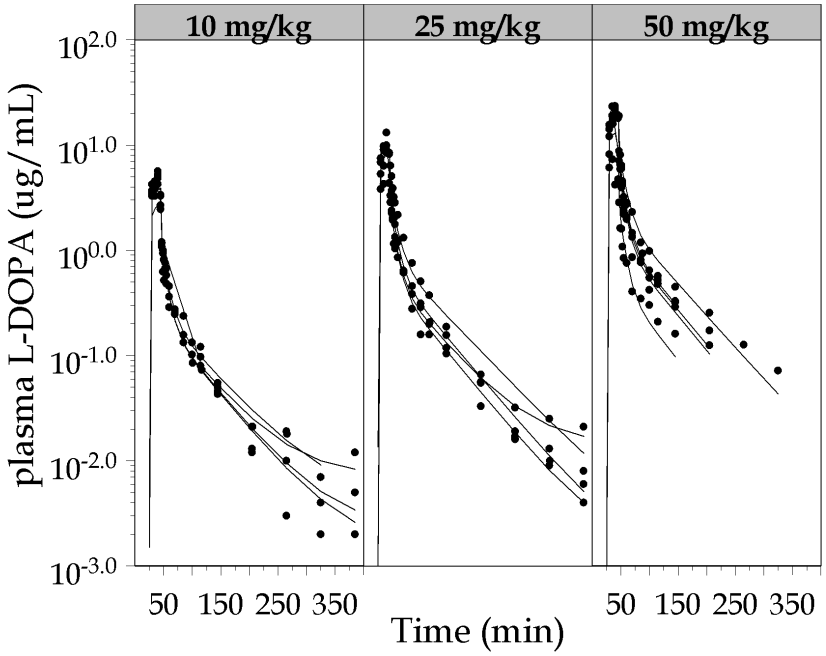

Figure 2: *L-DOPA concentration-time profiles in plasma, obtained after a 20-min i.v. infusion in Lewis rats. Depicted are the observed concentrations (dots), individual predictions (solid lines), separated by L-DOPA dose (in total 13 rats: 10 mg/kg: n=4; 25 mg/kg: n=4; 50 mg/kg: n=5).*

0.010 ± 0.004 pmol/mL and 0.024 ± 0.011 pmol/mL (mean ± SEM), respectively. Therefore, it can be concluded that no significant difference can be identified between the PK of L-DOPA in the untreated brain side *versus* the lesioned brain side.

DOPAC and HVA kinetic modelling

Striatal microdialysate baseline DOPAC levels in the lesioned brain side were about 6 times lower than in the untreated brain side and averaged 0.2 ± 0.19 pmol/mL and 1.3 ± 0.17 pmol/mL (mean ± SEM), respectively (P-value<0.01; Welch's t-test). Also, striatal microdialysate baseline HVA levels in the lesioned brain side were lower (approximately 4 times) than in the healthy brain side and averaged 0.25 ± 0.14 pmol/mL and 0.9 ± 0.08 pmol/mL (mean ± SEM), respectively (P-value=0.02; Welch's t-test). Figure 5 shows the population predicted microdialysate concentrations of DOPAC and HVA versus time for a typical rat per dose group. The goodness-of-fit plots for DOPAC and HVA in microdialysate are depicted in Figure 6. All structural parameters of the

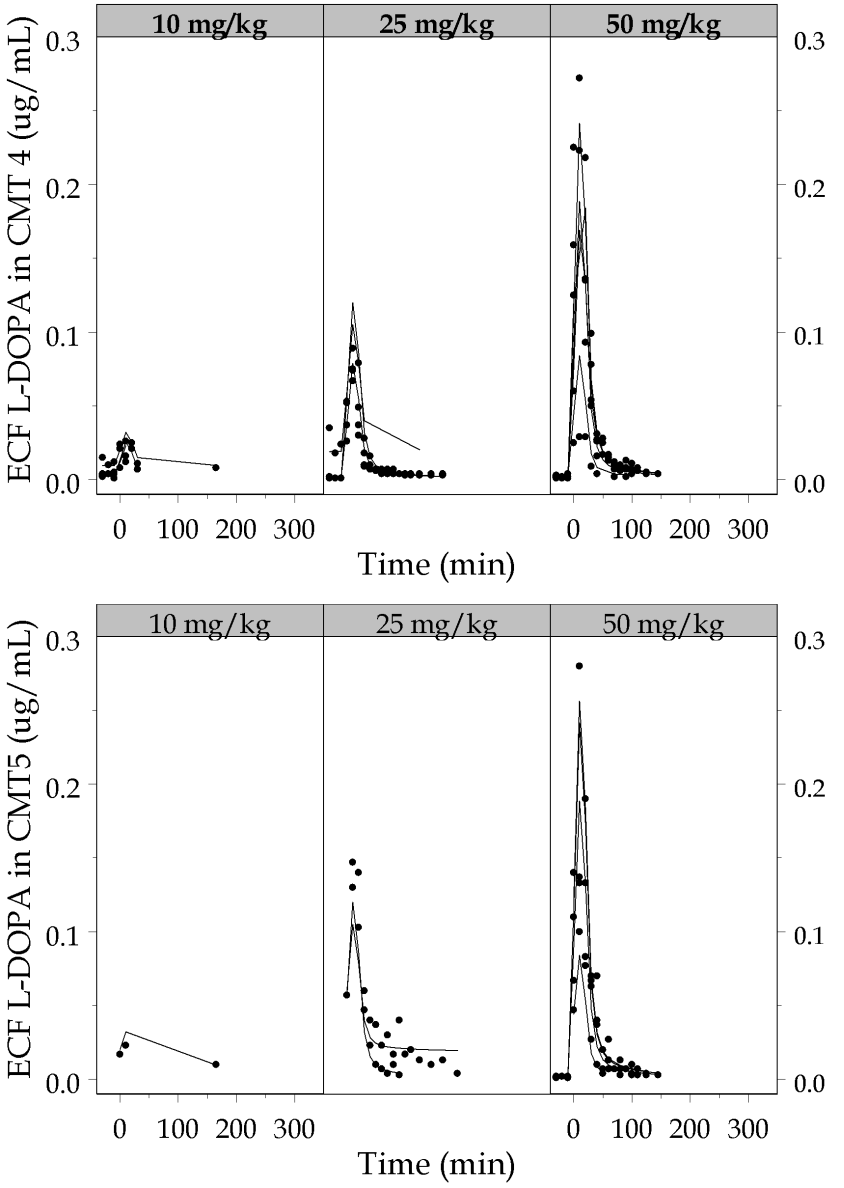

Figure 3: *L-DOPA concentration-time profiles in ECF in the untreated brain side (upper panel; in total 12 rats: 10 mg/kg: n=4; 25 mg/kg: n=3; 50 mg/kg: n=5) and in the lesioned brain side (lower panel; in total 7 rats: 10 mg/kg: n=1; 25 mg/kg: n=2; 50 mg/kg: n=4), obtained after a 20-min i.v. infusion in Lewis rats. Depicted are the observed concentrations (dots), individual predictions (solid lines), separated by L-DOPA dose.*

Table 1: *Population pharmacokinetic parameter estimates with the corresponding inter-individual coefficient of variation (CV%) and lower -and upper limit confidence intervals (LLCI and ULCI).*

Parameter	Estimate	CV%	LLCI-ULCI
L-DOPA			
Cl (mL/min)	30	21	17 – 43
ω^2 Cl	0.26	59	-0.041 – 0.56
V1 (mL)	98	39	24 – 172
V2 (mL)	157	15	112 – 202
V3 (mL)	599	15	425 – 773
V4 (mL)	13300	25	6810 – 19800
ω^2 V4	0.075	40	0.020 – 0.13
Q2 (mL/min)	22	24	11 – 32
Q3 (mL/min)	11	16	7.5 – 15
Q4 (mL/min)	22	14	16 – 29
Kin (min^{-1})	5.8	36	1.7 – 9.8
ω^2 Kin	0.94	41	0.19 – 1.7
Proportional error (plasma)	0.087	15	0.061 – 0.113
Proportional error (ECF)	0.17	21	0.10 – 0.24
UNTREATED BRAIN SIDE			
DOPAC			
k46 (min^{-1})	0.000044	28	0.000020 – 0.000068
ω^2 k46	0.50	60	-0.084 – 1.1
k40 (min^{-1})	0.53	23	0.29 – 0.77
ω^2 k40	0.42	46	0.045 – 0.80
k60 (min^{-1})	0.0053	17	0.0035 – 0.0071
ω^2 k60	0.19	58	-0.025 – 0.41
Residual error (additive)	0.0020	16	0.0014 – 0.0026
HVA			
k47(min^{-1})	0.000023	15	0.000016 – 0.000030
ω^2 k47	0.019	70	-0.0070 – 0.000030
k40 (min^{-1})	0.19	16	0.13 – 0.25
k70 (min^{-1})	0.0044	12	0.0033 – 0.0054
ω^2 k70	0.14	53	-0.006 – 0.28
Residual error (additive)	0.0028	24	0.0015 – 0.0041
LESIONED BRAIN SIDE			
DOPAC			
k58(min^{-1})	0.000054	51	-0.00000039 – 0.000011
ω^2 k58	1.0	40	0.23 – 1.9
k50(min^{-1})	0.36	55	-0.025 – 0.75
ω^2 k50	0.53	36	0.16 – 0.90
k80(min^{-1})	0.038	18	0.024 – 0.052
Residual error (additive)	0.0014	51	0.0000020 – 0.0027
HVA			
k59(min^{-1})	0.000016	42	0.0000028 – 0.000030
ω^2 k59	0.49	65	-0.14 – 1.12
k50(min^{-1})	0.14	55	-0.010 – 0.29
k90(min^{-1})	0.011	39	0.0027 – 0.020
Residual error (additive)	0.0034	41	0.00060 – 0.0061

Note: V5=V4, Q5=Q4 and k50=k40

population kinetic model for DOPAC as well as for HVA could be adequately estimated (Table 1). No dose-dependency was found in any of the parameters for DOPAC or HVA. For DOPAC, k46 and k58 (rate constants which describe the conversion of L-DOPA, via dopamine, to DOPAC) do not significantly differ, which means that there appears to be no effect of disease on the metabolism of L-DOPA via dopamine to DOPAC. The same can be said for HVA for which the values of k47 and k59 do not significantly differ. On the other hand, the elimination rate constants were found to be 7-fold and 2.5-fold higher in lesioned brain side compared to untreated side for DOPAC (k60 and k80) and HVA (k70 and k90), respectively.

Table 2: *Summary of goodness-of-fit based on the minimum value of objective function, of eight assumptions within the modelling of L-DOPA in plasma and brain*$_{ECF}$*. The assumption 'NONE' is where all parameters were estimated.*

Assumption	Results	*MVOF*	V4	V5	Q4	Q5	K40	K50
NONE	**general**	*-2814*	11100	16100	20.9	25.3	0.211	0.135
	SE		3840	6970	3.74	9.24	0.033	0.0132
	CV(%)		34.6	43.3	17.9	36.5	15.6	9.78
V5 = V4	**general**	*-2808*	12700		22.9	20.1	0.193	0.161
	SE		5700		4.33	7.13	0.0329	0.0185
	CV(%)		44.9		18.9	35.5	17	11.5
k50 = k40	**general**	*-2803*	13200	12000	20.1	26	0.175	
	SE		3870	4990	3.36	8.77	0.0226	
	CV(%)		29.3	41.6	16.7	33.7	12.9	
Q5 = Q4	**general**	*-2813*	11400	14400	22		0.211	0.135
	SE		3080	3700	3.18		0.027	0.0127
	CV(%)		27	25.7	14.5		12.8	9.41
V5 = V4 & k50 = k40	**general**	*-2804*	13000		21.4	24.3	0.179	
	SE		3410		3.2	5.56	0.0199	
	CV(%)		26.2		15	22.9	11.1	
V5 = V4 & Q5 = Q4	**general**	*m.t.*	13000		22		0.19	0.17
	SE		-	-	-	-	-	-
	CV(%)		-	-	-	-	-	-
k50 = k40 & Q5 = Q4	**general**	*-2801*	13900	10600	21.7		0.173	
	SE		8290	6610	7.79		0.0276	
	CV(%)		59.6	62.4	35.9		16	
V5 = V4 & k50 = k40 & Q5 = Q4	**general**	*-2801*	13300		22.4		0.175	
	SE		3310		3.17		0.0201	
	CV(%)		24.9		14.2		11.5	

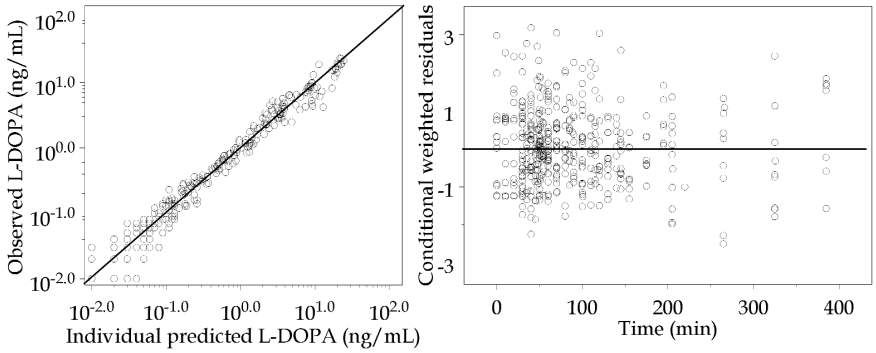

Figure 4: *Goodness-of-fit plots of L-DOPA concentrations in plasma and brainECF as described by the model depicted in Figure 1. The left panel depicts a scatterplot of the observed L-DOPA concentrations versus the individual model predictions. The line represents the line of unity. The right panel depicts a scatterplot of the conditional weighted residuals versus time.*

4. Discussion

In this study the relationship between plasma and $brain_{ECF}$ kinetics of L-DOPA and its conversion into the dopamine metabolites DOPAC and HVA was measured in rats, in parallel in the lesioned and untreated brain side, at 14 days post-rotenone injection. NONMEM was used to develop a population based PK model. Basal concentrations of DOPAC and HVA in striatal microdialysate differed between the lesioned and the untreated brain side. Furthermore, the model showed that without changes in BBB transport of L-DOPA, disease-related changes were observed in the kinetics of these dopamine metabolites following L-DOPA administration.

The results described in this paper are the first in which both plasma PK and $brain_{ECF}$ PK of L-DOPA in untreated and diseased (lesioned) conditions are described by one population PK model. The PK of L-DOPA in plasma following *i.v.* administration was best described by a three-compartmental model, like previously reported (Doller *at al*., 1978). In our study, we were not able to measure endogenous plasma concentrations of L-DOPA (LLQ was 1 µg/mL). Sato *et al* (1994a) found in their study in rats a basal level of 2.1 ± 0.6 mg/L. In our study, rats were fasted overnight which might be the reason for much lower endogenous L-DOPA in plasma. The total clearance of exogenous plasma in the study by Sato *et al* (1994a) was 3.13 L/h/kg which is in the same range as our value of 6.25 L/h/kg (Cl= 30 mL/min, Table 1; mean weight of the rats before start of the experiment was 288 ± 13g).

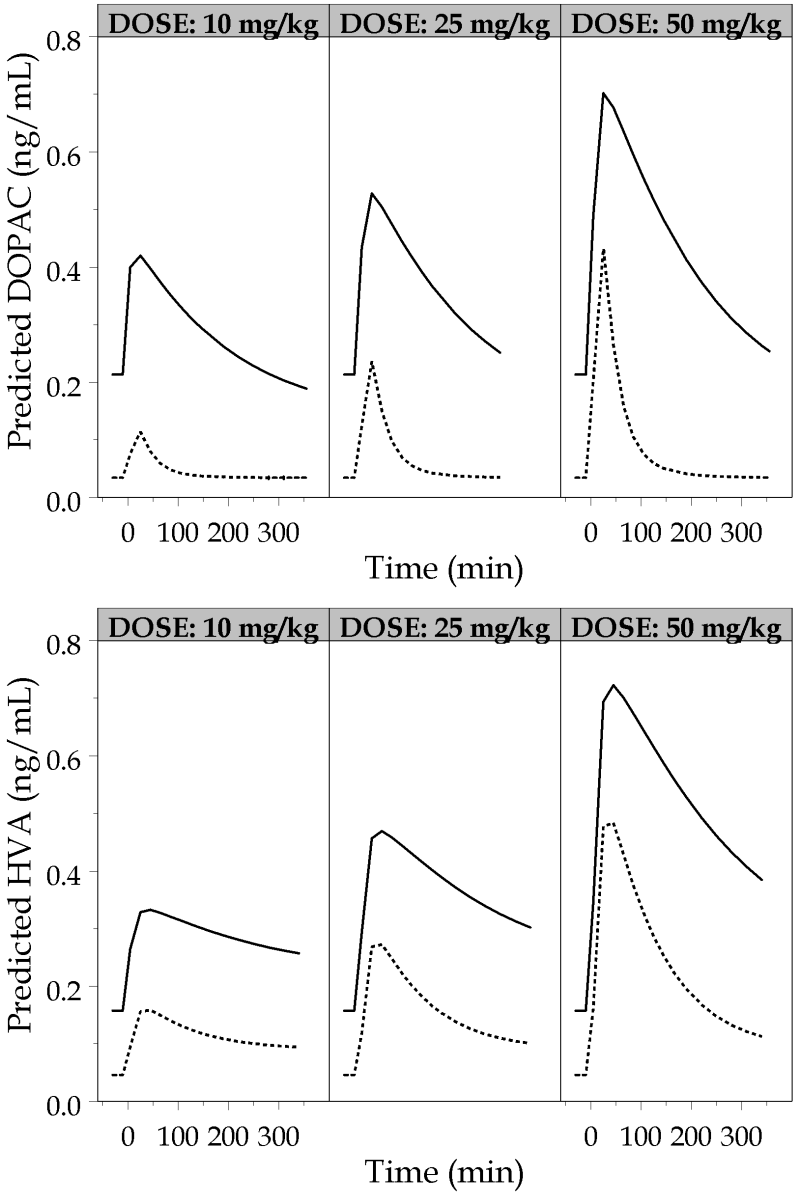

Figure 5: *Depicted are the population predicted concentrations of DOPAC (upper panel) and HVA (lower panel) in the untreated brain side (——) and lesioned brain side (- - -) according to the model as described in Figure 1, separated by L-DOPA dose. For each dose, a typical rat is depicted.*

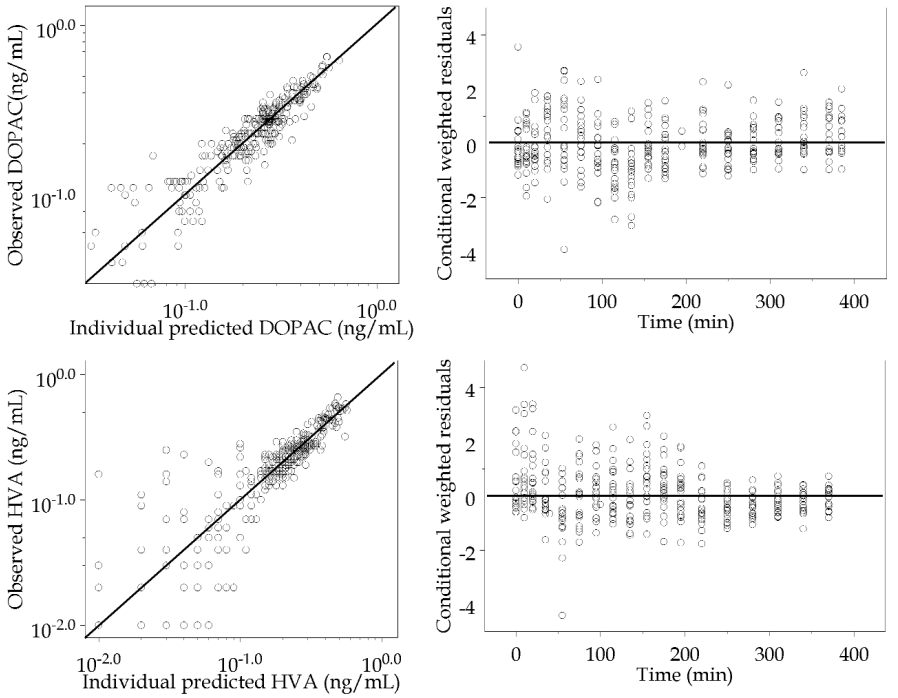

Figure 6: *Goodness-of-fit plots of DOPAC (upper panel) and HVA (lower panel) concentrations in microdialysate as described by the model depicted in Figure 1. The left graph of each panel depicts a scatterplot of the observed dialysate concentrations versus the individual model predictions. The line represents the line of unity. The right graph of each panel depicts a scatterplot of the weighted residuals versus time.*

In our study, following the rotenone infusion in the MFB, Parkinson's disease state was defined as a density of TH immunostaining in the striatum that was less than 40% of control (responders). Data indicated that the success of the model to induce neurodegeneration was similar to our previous study (Ravenstijn *at al.*, 2008), and the disease pathology was induced in about 70% of the rats. No difference in BBB transport of L-DOPA between the untreated and lesioned brain side were found. Also, our results did not indicate a disease-induced change in the endogenous production of L-DOPA in the brain (Kin; 5.8 min^{-1}). Since dopamine-producing neurons are diminished in Parkinson's disease (Dauer and Przedborski, 2003), one would expect a decrease in the endogenous concentration of L-DOPA, as it is a product of the metabolism of L-tyrosine by TH. However, it has been reported that TH enzyme activity, as a function of the degree of dopamine loss, is upregulated in the striatum of 6-OHDA lesioned rats and of

MPTP-treated rhesus monkeys (Pifl and Hornykiewicz, 2006). This phenomenon is assumed to be a compensatory mechanism of the remaining dopaminergic neurons triggered by the synaptic dopamine loss (Rose *at al.*, 1989). Upregulation of TH in the rotenone lesioned brain side seems therefore to explain our results.

Furthermore, the metabolism of L-DOPA via dopamine to DOPAC in the untreated brain (0.000044 min^{-1}) was indistinguishable from this metabolism in the lesioned brain side (0.000054 min^{-1}). Also, the metabolism of L-DOPA via dopamine to HVA in the untreated brain side (0.000023 min^{-1}) was similar to that in the lesioned brain side (0.000016 min^{-1}). These relatively low values, reflecting a slow conversion of L-DOPA via dopamine to these metabolites, is due to dopamine being the main metabolite of L-DOPA in the early phase after drug administration (Miwa *at al.*, 1992). Furthermore, this newly synthesised dopamine is localised exclusively in the intracellular compartment within this timeframe (DeJesus *at al.*, 2000; Miwa *at al.*, 1992). It is therefore not readily available for metabolism to DOPAC and/or HVA. Overall, in contrast to our expectations, it can be said that the disease conditions at 2 weeks post-rotenone-injection in the MFB did not result in any change in the kinetics of L-DOPA.

Differences for the dopamine metabolites DOPAC and HVA between lesioned and untreated brain side were observed for baseline concentrations as well as for elimination rate constants following L-DOPA administration, while their formation rate constants remained unaffected. The baseline concentrations of DOPAC and HVA were about 10-fold decreased in the lesioned brain side (Figure 5). These findings are similar to what has been reported in other studies in rats, after an intracerebral injection of rotenone into the MFB (Antkiewicz-Michaluk *at al.*, 2004), and intracerebral 6-OHDA injection (Brannan *at al.*, 1990; Sarre *at al.*, 1992). Such concentrations result from the rate of formation relative to that of elimination. As no changes in formation rate constants of DOPAC and HVA were found in the lesioned relative to the untreated brain side, decrease in baseline concentrations were the result of solely the increased elimination rate constants.

In our model, the metabolism from dopamine to DOPAC and HVA was explained by first-order kinetics (Figure 1, Table 1), like in the model previously developed (Sato *at al.*, 1994a; Sato *at al.*, 1994b). Following L-DOPA administration, for DOPAC, the elimination rate constant in the lesioned brain side (0.038 min^{-1}) was increased about 7-fold compared with the elimination rate constant in the untreated side (0.0053 min^{-1}). For HVA this disease-induced increase was about a factor 2.5 (0.011 min^{-1} for the lesioned versus 0.0044 min^{-1} for the untreated brain

side). Elimination of DOPAC from the ECF may occur by conjugation, in rats mostly to sulphates (Swahn and Wiesel, 1976) or by transformation to HVA by COMT. HVA is formed as metabolite of DOPAC but also directly from dopamine (Mannisto *at al.*, 1992; Westerink and de Vries, 1985; Wood and Altar, 1988). The mechanisms of elimination of HVA from the brain are not fully clear. HVA may leave the brain by passive diffusion (Amin *at al.*, 1992). Also, HVA effluxes from the brain via a probenecid-sensitive organic anion transport (OAT3) system, present at the BBB (Mori *at al.*, 2003). This HVA efflux transport system is likely to play an important role in controlling the level of HVA in the CNS. The apparent in vivo efflux rate constant of HVA from the brain was determined by (Mori *at al.*, 2003) to be 0.017 min^{-1}. Our value for the elimination rate constant of HVA in the untreated brain side was 0.044 min^{-1} and is in the same order of magnitude. The increased values that we found for the elimination rate constants for DOPAC and HVA could have been the result of upregulation of one of the active elimination processes. Though expressions of OATs are affected (mainly downregulated) in certain renal and hepatic diseases (Anzai *at al.*, 2006), to our knowledge, no studies have been performed indicating changes in OAT functionality at the BBB. However, even without the explanation of changes in functionality of the eliminating processes, the lower baseline values and increased elimination rates of DOPAC and HVA can be explained. In our study, the rate of formation of DOPAC and HVA is much lower than their rates of elimination (Table 1). The concept of "absorption-rate limited elimination" (Rowland and Tozer, 1995), that may occur when the absorption rate constant is significantly lower than the (true) elimination rate constant, might apply to our situation. In "absorption-rate limited elimination", the absorption process is very slow so that the product of the absorption rate constant and amount remaining to be absorbed approximately equals the product of the elimination rate constant and the amount to be eliminated ($k_a.A_a \sim k_{el}.A_{el}$). We propose for our results a "formation rate-limited elimination". Assuming identical distribution volumes for dopamine and its metabolites, the amounts can be divided by the distribution volume to give concentrations. As the formation rate constants of DOPAC and HVA are much lower than their corresponding elimination rate constants, the actual concentration of dopamine ("remaining to be metabolised") will govern the formation rate, and therewith the elimination rates of DOPAC and HVA. Then, $k_{form\ metabolite} * C_{dopamine} \sim k_{elim\ metabolite} * C_{metabolite}$. With unchanged values for formation of the metabolites ($k_{form\ metabolite}$ or k46 and k58 for DOPAC and k47 and k59 for HVA; see table 1), and lower baseline concentrations as measured

for both DOPAC and HVA, a higher (apparent) elimination rate constant is observed if dopamine concentrations would be lower in the lesioned brain side. This is indeed plausible with a diminished amount of dopaminergic neurons. This indicates that, though we have not been able to measure dopamine directly, the clearly lower baseline concentrations of DOPAC and HVA found in the lesioned brain side together with the increased values for the elimination rate constants, provides insight in changes in dopamine concentrations by the disease process.

We are the first to have developed a population PK model on the kinetics of L-DOPA, and its conversion on DOPAC and HVA in both untreated and (Parkinson's) lesioned brain. This opens up the possibility to further investigate the potential changes in the PK and conversion of L-DOPA at different stages of Parkinson's disease. Then, further investigations may focus on a potential relationship between a gradual decline in %TH staining and DOPAC and HVA microdialysate concentrations, which would give the opportunity to *in vivo* determine the Parkinson's disease state at multiple days as there is no need to sacrifice the experimental animal.

In conclusion, our study demonstrates that 2 weeks following a unilateral infusion of rotenone into the rat brain, there are no changes in the kinetics of L-DOPA in the lesioned hemisphere of the brain. However, a clear effect of dopamine depletion on the levels and elimination rates of DOPAC and HVA in brain was observed. This provides indirect information (confirmed by TH immunohistochemistry) for decreased dopamine concentrations at the lesioned brain side in accordance with "formation rate-limited elimination" PK principles. The models used here may help to unravel the molecular and biochemical mechanisms beyond the changes in the PK and its relation to the conversion of L-DOPA, in different stages of Parkinson's Disease. Further studies in other Parkinson's disease models that show progressive degeneration over time are warranted.

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Section III :
Conclusions and
General Discussions

Chapter 6

Summary, Conclusions and Perspectives

1. General objective

The objective of the research described in this thesis was to explore rotenone as a toxin for inducing Parkinson's disease in rats as a new rat model of this disease, and to use this rat model in pharmacokinetic(-pharmacodynamic) (PK-PD) studies on antiparkinson drugs with special reference to blood-brain barrier (BBB) functionality.

2. Understanding drug response in Parkinson's disease: the role of the BBB

In most neuropharmacological studies the effect of drugs in the CNS are still related to the dose and the mechanisms which may affect the disposition (e.g. absorption, distribution, metabolism and excretion) and thereby ultimately the drug response is not considered. Different factors like genetics, species, gender, age, environmental and pathological conditions can influence the drug response. Parkinson's disease is a progressive neurodegerative disease that lacks good treatment, especially at later stages. Apart from plasma pharmacokinetics, mechanisms that govern CNS drug distribution and response include the rate and extent of BBB transport and the kinetics of distribution within the brain including the brain target distribution. For the development of new drugs as well as for the optimisation of therapy with the current drugs, the variability of these individual mechanisms and contribution in terms of rate and extent should be investigated. **Chapter 2** summarised the mechanisms and sources of variation in the response to drugs used in the treatment of Parkinson's disease. As the BBB is a key player in the relationship between plasma and brain pharmacokinetics, the influences of disease states on BBB functionality in the various stages of the disease is important in order to judge on drug effects. To that end, more integrative research approaches are needed. This warrants the application of a systems pharmacology approach in investigations on variability in drug response in Parkinson's disease.

3. Animal models as a tool in systems pharmacology research on Parkinson's disease treatment

For the development of mechanism-based PK-PD models, animal models are essential. An animal model for Parkinson's disease displaying the slow progressive nature of the disease would provide the biological system-specific parameters needed to determine the neuroprotective properties of new drugs, preclinically. In **Chapter 3**, we presented an overview of the currently available

animal models of Parkinson's disease, with their main characteristics, followed by a summary of available behavioural tests and a discussion on the microdialysis technique for the assessment of BBB functionality. The use of a chronic animal model for Parkinson's disease is a first step in the characterisation of drug effects on disease processes and disease progression. A technique such as intracerebral microdialysis may be applied to determine extracellular, unbound concentrations of endogenous compounds (e.g. biomarkers for Parkinson's disease or BBB functionality) as well as of exogenous compounds (e.g. antiparkinson drugs or marker compounds for specific transport mechanisms of the BBB). Furthermore, behavioural tests may also be applied as a pharmacodynamic read-out. The data obtained from these experiments give information on processes on the causal path between drug administration and response (e.g. target site distribution, target binding and activation, transduction/homeostatic feedback and diseases processes/progression). With this, mechanism-based PK-PD models can be developed which can have properties for extrapolation and prediction in systems pharmacology research (Danhof *et al.*, 2007).

4. The exploration of rotenone as a toxin for inducing Parkinson's disease in rats

In **Chapter 4**, two methods of inducing Parkinson's disease in rats are introduced and compared using the neurotoxin rotenone. The administration of low-dose intravenous or subcutaneous rotenone to rats had previously been shown to produce a slow, selective degeneration of nigrostriatal dopaminergic neurons accompanied by the formation of α-synuclein-positive LB-like inclusions as seen in Parkinson's disease (Betarbet *et al.*, 2000; Sherer *et al.*, 2003). Rotenone's advantages of being able to create an animal model exhibiting a slow progression of disease and the formation of LB-like structures outweighed the use of the well-documented but more acute neurotoxins 6-OHDA and MPTP. Ultimately, this animal model would be applied as a tool for mechanism-based PK-PD disease progression models, in which time-dependent changes in the biological system of diseased animals are taken into account (Post *et al.*, 2005).

The subcutaneous route was chosen as this was less labor intensive and produced the same results as administration via jugular vein cannulation (Sherer *et al.*, 2003). Studies were performed in which we investigated the effect of subcutaneous rotenone on bodyweight, behaviour and BBB permeability with sodium fluorescein as a marker and intracerebral microdialysis as a tool to assess

intracerebral fluorescein concentrations. The intracerebral microdialysis technique offers the advantage of allowing repeated or continuous sampling in freely moving animals. Herewith, it provides important information on the BBB transport and brain distribution. Post-mortem analysis consisted of assessing nigrostriatal damage based on immunohistological staining with TH as well as peripheral organ pathology. The results indicated that subcutaneously administered rotenone failed to produce dopaminergic lesions in the SNc and striatum and, moreover, led to extensive peripheral organ toxicity. BBB permeability for fluorescein following subcutaneously administered rotenone was changed, however due to peripheral toxicity. Other labs have shown similar results on systemically administered rotenone (Fleming *et al.*, 2004; Hoglinger *et al.*, 2003; Lapointe *et al.*, 2004; Zhu *et al.*, 2004).

These results led to the development of the method in which rotenone was administered directly into the median forebrain bundle (MFB) in the brain to be able to overcome any peripheral toxicity. Intracerebrally-administered rotenone (MFB or SNc) is able to decrease striatal and nigral dopamine and its metabolites (Antkiewicz-Michaluk *et al.*, 2004; Saravanan *et al.*, 2005) and to induce behavioural changes similar to Parkinson's disease (Sindhu *et al.*, 2006). An infusion into the MFB was chosen as this might develop a slower, more progressive degeneration of dopaminergic cells as compared to an intranigral infusion (Sindhu *et al.*, 2005). To determine the "optimal" dose for inducing Parkinson's disease in rats, three different doses of rotenone were tested (0.5, 2.0 and 5.0 µg). Here, we monitored bodyweight and performed post-mortem peripheral organ toxicology to confirm the absence of any influence of the rotenone peripherally. Additionally, BBB permeability was assessed with sodium fluorscein as a marker and intracerebral microdialysis. In a separate experiment rotational behaviour was assessed using amphetamine in animals receiving the highest rotenone dose (5.0 µg). Post-mortem analysis using TH on the striatum and SNc was performed. The SNc were analysed for α-synuclein inclusions. Results showed a progressive lesion of the nigrostrial dopaminergic pathway with no associated peripheral toxicity. Furthermore, a large increase in amphetamine induced rotational behaviour was seen and a few rats showed α-synuclein immunoreactivity and aggregation. However, no changes in passive BBB permeability were detected. The results indicated that rotenone infused intracerebrally (specifically the 5.0 µg-dose) is able to create a progressive rat model for Parkinson's disease, which could then be used in PK-PD and other types of experiments.

5. The intracerebral rotenone model of Parkinson's disease in rats: altered conversion of L-DOPA into DOPAC and HVA without changes in BBB transport

In **Chapter 5**, the relationship between plasma and $brain_{ECF}$ kinetics of L-DOPA and its effect on dopamine metabolites DOPAC and HVA was measured in the untreated as well as in the brain side 14 days after an injection of rotenone. For this purpose rotenone (5 μg) was infused unilaterally into the MFB to induce Parkinson's disease in Lewis rats as presented in **chapter 4**. The contralateral, non-infused brain side was used as control. Three groups were used in the experiment, each receiving a different dose of L-DOPA (10, 25 or 50 mg/kg). Plasma samples were collected to determine plasma PK of L-DOPA and (dual-probe) intracerebral microsialysis was used as a tool to measure extracellular concentrations of L-DOPA, DOPAC and HVA in the striatum of both the lesioned and control/untreated brain sides. This enabled us to compare the diseased brain concentrations to the untreated side. Dopamine could not be detected as the concentrations were below the limit of quantification. Post-mortem analysis using TH immunostaining on the striatum was performed. These data were used to determine "responders" to rotenone, which had a TH staining percentage of 40% or lower and were considered to be diseased. NONMEM was used to develop a population based PK model. The results described in this chapter are the first in which both plasma and $brain_{ECF}$ PK of L-DOPA under untreated and diseased conditions are described by one population PK model. The results indicated that the disease conditions at 2 weeks post-rotenone-injection in the MFB did not result in any change in the kinetics of L-DOPA. Merely, a clear effect of disease on the levels and elimination rates of DOPAC and HVA in brain were found, providing indirect information on decreased dopamine concentrations at the diseased brain side based on flip-flop kinetic principles.

6. Discussion and future perspectives

The objective of the research described in this thesis was to explore rotenone as a toxin for inducing Parkinson's disease in rats as a new rat model of this disease, and to use this rat model in PK(-PD) studies on antiparkinson drugs with special reference to BBB functionality in the context of disease.

The results indicated that subcutaneously administered rotenone failed to produce dopaminergic lesions and led to extensive peripheral organ toxicity. BBB permeability for fluorescein following subcutaneously administered rotenone

was changed, however due to peripheral toxicity. Other labs have shown similar results on systemically administered rotenone (Fleming *et al.*, 2004; Hoglinger *et al.*, 2003; Lapointe *et al.*, 2004; Zhu *et al.*, 2004). Rotenone infused intracerebrally (specifically the 5.0 µg-dose into the MFB) is, however, able to create a progressive rat model for Parkinson's disease, which could be used in PKPD and other types of experiments. We showed in a few cases α-synuclein immunoreactivity and aggregation. These have not been observed in previous experiments using an intracerebral infusion of rotenone (Alam *et al.*, 2004; Antkiewicz-Michaluk *et al.*, 2004; Saravanan *et al.*, 2005; Sindhu *et al.*, 2005). Furthermore, the decrease in striatal TH staining seems to reach a minimal plateau at 28 days post-injection. Further experiments to follow the progression at longer post-injection intervals would be required to evaluate if there is disease progression and further α-synuclein development beyond 28 days. Many studies have demonstrated substantial and important age-related changes in neurochemistry and neurobiology. Data on the decreases and alterations in the dopaminergic neurotransmitter pathway have been relatively consistent in both animal and human studies (Adolfsson *et al.*, 1979; Bucht *et al.*, 1981; Wenk *et al.*, 1989). Also, age seems to increase the sensitivity of dopaminergic neurons to rotenone toxicity in rats (Phinney *et al.*, 2006). These findings indicate to further investigate this for the intracerbrally infused rotenone rats and to study the influence that age might have on the development of α-synuclein inclusions.

In our intracerebral microdialysis studies using sodium fluorescein as a marker for BBB permeability, we did not demonstrate any changes in striatal BBB permeability. We performed our microdialysis study with sodium fluorescein at one particular time point at a specific location in the brain (striatum), and it therefore cannot be concluded that no changes in BBB permeability would occur in other stages of the disease, or at other places within the brain such as the SNc. Carvey and collegues (Carvey *et al.*, 2005) have found leakage of FITC in the SNc and striatum which was always patchy in appearance. This suggests that the 6-OHDA lesion which they applied in their studies, leads to multiple focal breakdowns in the BBB function. It is worthwhile investigating this phenomenon in our animal model at different timepoints after the injection of rotenone into the MFB. The evidence in literature for alterations in BBB functionality in Parkinson's disease is still growing and shows that BBB research in this disease needs more future attention. Parkinson's disease patients have shown to have an increase in vascular density in the SNc, but not the ventral tegmental area (Faucheux *et al.*,

1999), and a reduced P-gp function was found using PET to measure brain uptake of [^{11}C]-verapamil (Bartels *et al.*, 2008; Kortekaas *et al.*, 2005). A rat model using 6-OHDA has demonstrated that dyskinesias in animals were associated with increased entry of L-DOPA into the striatum (Carta *et al.*, 2006; Westin *et al.*, 2006) which was also seen using PET imaging in patients with peak-dose dyskinesias at 1 hour after L-DOPA administration (Fuente-Fernandez *et al.*, 2004). The dyskinetic 6-OHDA rats also exhibited a significant increase in total blood vessel length and a visible extravasation of serum albumin in the SNc (Westin *et al.*, 2006), indicating a role for the BBB in the altered transport of L-DOPA to the brain.

In our studies using the intracerebral rotenone model no change in the plasma kinetics, nor in the brain distribution kinetics of L-DOPA was seen at 2 weeks post-rotenone-injection. However, a clear effect of disease on the levels and elimination rates of DOPAC and HVA in brain were found, providing indirect information on decreased dopamine concentrations at the diseased brain side based on flip-flop kinetic principles. The percentage of intact TH staining found in the rotenone-treated hemisphere compared to the untreated hemisphere was below 40% in 12 out of 17 rats used in this experiment, and higher than 90% in the remaining 5 rats. Further experiments might be directed towards investigating the kinetics of L-DOPA in rats with more advanced lesions. Bromocriptine, a D2 antagonist used in the treatment of Parkinson's disease, has shown to be a substrate for P-gp (Vautier *et al.*, 2006). Given the evidence that P-gp function might be altered in Parkinson's disease (Bartels *et al.*, 2008; Kortekaas *et al.*, 2005), it would be worthwhile to investigate possible changes in the pharmacokinetics (and pharmacodynamics) of bromocriptine in the intracerebral rotenone rat model. Another D2 receptor agonist used in the treatment of Parkinson's disease is pramipexole which is a cationic drug which not only crosses the BBB by diffusion but also via organic cation-sensitive transporter (Okura *et al.*, 2007). This drug might be an interesting model drug to investigate Parkinson's disease related changes in this BBB transporter.

7. Conclusion

In the intracerebral rotenone model of Parkinson's disease, studies were performed at day 14 after injection of rotenone. At this time-point no changes were found in passive BBB permeability, nor in BBB transport modes of L-DOPA (LAT transporter & passive permeability). However, a diseased condition was

present as indicated by the clear effect of rotenone on the levels and elimination rates of DOPAC and HVA in brain that provided information on decreased dopamine concentrations at the diseased brain side.
Altogether it was concluded that the intracerebral infusion of rotenone into the MFB is able to create a chronic and progressive rat model for Parkinson's disease, which is suitable as a tool in systems pharmacology research on Parkinson's disease.

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Nederlandse Samenvatting

1. Inleiding

Doel van dit onderzoek

Het doel van de studies beschreven in dit proefschrift was te onderzoeken of het gebruik van het neurotoxine rotenon in ratten geschikt was voor het induceren van een meer chronisch en progessief verloop van de ziekte van Parkinson ten behoeve van onderzoek naar de relatie tussen farmacokinetiek (PK) en farmacodynamiek (PD) van geneesmiddelen die worden gebruikt voor de behandeling van de ziekte van Parkinson. Hierbij werd speciaal de nadruk gelegd op mogelijke wijzigingen in snelheid en mate van het transport over de bloed-hersenbarrière (BHB) ten gevolge van de pathologische condities.

De ziekte van Parkinson

De ziekte van Parkinson is een langzaam voortschrijdende ziekte waarbij dopamine-producerende zenuwcellen in het striatum en de substantia nigra van de hersenen afsterven (neurodegeneratie). Met de tijd neemt hierdoor de concentratie van dopamine in de hersenen af, met name in het striatum. Door dit dopamine-tekort gaan de hersenkernen -die belangrijk zijn voor met name het uitvoeren van bewegingen- slechter functioneren. In de nog aanwezige dopaminerge zenuwcellen ontwikkelen zich ophopingen van het eiwit alfasynucleïne. Dergelijke ophopingen worden Lewy bodies genoemd.

De klinische symptomen die optreden bij de ziekte van Parkinson hebben voornamelijk betrekking op het bewegingsapparaat (motorisch systeem), te weten rigiditeit (stijfheid van de ledematen), akinesie (langzaam en weinig bewegen), rusttremor (trillen bij rust), en gestoorde (voorovergebogen) houding en houdingsreflexen. Naast deze motorische verschijnselen, kunnen er ook symptomen van het autonome zenuwstelsel optreden (o.a. overmatige transpiratie, obstipatie), alsmede psychische verschijnselen (depressie, vermoeidheid en dementie).

De behandeling van de ziekte van Parkinson bestaat uit het aanvullen van het tekort aan dopamine in de hersenen. Dit kan niet door het toedienen van dopamine zelf, aangezien dopamine de bloed-hersenbarrière (BHB) niet kan passeren.

De precursor (uitgangsstof) van dopamine, het L-3,4-dihydroxyphenylalanine (L-DOPA), kan wel in de hersenen terecht komen alwaar het door de nog aanwezige dopaminerge zenuwcellen omgezet wordt in dopamine. Anderszins kunnen dopamine agonisten de werking van dopamine nabootsten. Echter tot op

heden is de therapie van L-DOPA in combinatie met een decarboxylase remmer de meest gebruikte in de behandeling van de ziekte van Parkinson.

L-DOPA wordt via een actieve transporter over de BHB naar de hersenen getransporteerd. Zoals eerder gezegd neemt bij het voortschrijden van de ziekte het aantal zenuwcellen in de substantia nigra steeds verder af. Aangezien de dopaminerge zenuwcellen verantwoordelijk zijn voor de omzetting van L-DOPA naar dopamine, onstaat er in een verder gevorded stadium van de ziekte van Parkinson een probleem met betrekking tot de effectiviteit van de L-DOPA therapie. Er is gebleken dat de werking van L-DOPA minder constant wordt. Ten opzichte van het vroege stadium van de ziekte is in het late stadium na toediening van L-DOPA enerzijds eerst sprake van ongewenste effecten -zoals een teveel aan onwillekeurige bewegingen (dyskinesieën)-, terwijl anderzijds de werkingsduur veel korter is of soms zelfs afwezig ("off-verschijnselen"). Om deze ongewenste effecten te vermijden wordt L-DOPA in latere stadia van de ziekte veelal gegeven in combinatie met een dopamine agonist of met een catechol-O-methyltransferase (COMT) remmer of een monoamine-oxidase-B (MAO-B) remmer. Dopamine agonisten hebben het voordeel dat ze een langere werkingsduur hebben (langere halfwaardetijd) in vergelijking tot L-DOPA. Remmers van de enzymen COMT en MAO zorgen ervoor dat L-DOPA minder snel wordt afgebroken waardoor L-DOPA langer in effectieve concentraties aanwezig kan zijn. Desalniettemin zal, in een verder gevorderd stadium van de ziekte, de werking van antiparkinson geneesmiddelen verminderen en zullen er meer complicaties in de motoriek optreden. Dit betekent dat er sterke behoefte bestaat aan betere middelen voor de behandeling van de ziekte van Parkinson. De toekomst van het geneesmiddelenonderzoek voor de ziekte van Parkinson richt zich dan ook met name op de ontwikkeling van geneesmiddelen met een beschermende of herstellende werking op de neurodegeneratie.

De bloed-hersenbarrière

Geneesmiddelen voor de behandeling van de ziekte van Parkinson hebben hun werking in de hersenen en dienen hiervoor de BHB te passeren. De BHB wordt gevormd door de wand van de hersencapillairen. Door zogenaamde "tight junctions" zijn deze hersenendotheelcellen zeer nauw aaneengesloten. De BHB beschikt over verschillende transportmechanismen. Eén van de transportmechanismen is passief, door middel van diffusie. Dit kan gebeuren tussen de cellen door (paracellulair) zowel als door de membranen van de cellen

heen (transcellulair). Kleine, lipofiele en ongeladen stoffen kunnen via de transcellulaire route de BHB passeren, terwijl grotere, hydrofiele stoffen dit eerder via de paracellulaire route doen, die zeer beperkt is door de tight junctions. De hersenendotheelcellen bevatten ook een aantal specifieke transporter-eiwitten die actieve influx (bloed naar hersenen) en efflux (hersenen naar bloed) verzorgen. De influx transporters zorgen voor de opname van stoffen die essentieel zijn voor een goede werking van de hersenen. Zo zijn er transporters specifiek voor de opname van eiwitten, peptides, glucose, aminozuren, vitamines en hormonen. Ook lichaamsvreemde stoffen, waaronder geneesmiddelen, kunnen afhankelijk van hun fysisch-chemische eigenschappen via deze transporters in de hersenen opgenomen worden. Zo wordt L-DOPA in de hersenen opgenomen via de grote aminozuur transporter (LAT1). Efflux transporters zorgen voor het verwijderen van toxische stoffen en afvalstoffen uit de hersenen en kunnen zo ook diverse geneesmiddelen uit de hersenen verwijderen. De meest bekende efflux transporter is het P-glycoproteïne (P-gp). De substraten van het P-gp zullen door de werking van het P-gp lagere concentraties in de hersenen bereiken. Verder is er naast de BHB is ook een barrière te vinden tussen het bloed en de hersenvloeistof (liquor). De BHB wordt echter gezien als de belangrijkste barrière voor stoffen om de hersenen te bereiken.

2. Mechanismen in geneesmiddelwerking in de ziekte van Parkinson: BHB in de hoofdrol

Het netto transport van een bepaald geneesmiddel door de BHB, met als resultaat een bepaalde concentratie van dat geneesmidel in de hersenen, wordt bepaald door de som van alle BHB transportmechanismen voor deze stof. Veranderingen in één of meerdere BHB transportmechanismen kunnen de PK van dit geneesmiddel in de hersenen veranderen, dus ook de werking van het geneesmiddel. Het functioneren van de BHB kan variëren met verschillende fysiologische en pathologische omstandigheden of als gevolg van chronisch geneesmiddel gebruik. Zo is op hogere leeftijd met name de werking van specifieke transportmechanismen voor o.a. choline, glucose en peptides verminderd. Ook wordt er gedacht dat neurodegeneratie gerelateerd is aan de integriteit van de BHB. Zo zijn er aanwijzingen gevonden voor een aangetaste BHB in hersenbiopten van patienten met de ziekte van Alzheimer, de meest voorkomende neurodegeneratieve ziekte. Daarnaast is in een kleine groep patienten met de ziekte van Parkinson met Positron Emissie Tomografie de

werking van het P-gp in de BHB gemeten die aanwijzingen gaven voor een verminderde werking van het P-gp. Verder zijn er enkele studies in diermodellen die duiden op een veranderde functie van de BHB; één daarvan specifiek in het striatum en de substantia nigra. Aangezien de centrale werking van een geneesmiddel nauw samenhangt met het concentratie in de hersenen en de BHB de relatie tussen plasma concentratie en hersenconcentratie bepaalt, zal BHB transport in verschillende stadia van de ziekte van Parkinson onderdeel moet uitmaken van studies naar de effectiviteit van antiparkinson middelen.

In neurofarmacologisch onderzoek wordt de werking van een geneesmiddel veelal nog slechts gerelateerd aan de dosis. Daarbij wordt voorbijgegaan aan de invloed van specifieke mechanismen die de werking van het geneesmiddel in de hersenen bepalen. Dit zijn met name plasma PK en eiwitbinding, het BHB transport, de verdeling van de stof binnen de hersenen zelf en naar de plaats van werking (aangrijpingspunt van het geneesmiddel), alsook de interactie met het aangrijpingspunt. Deze mechanismen zullen niet in alle omstandigheden een evengrote bijdrage hebben aan het werkingsprofiel van het geneesmiddel. Verschillen in factoren zoals genetische achtergrond, soort, geslacht, leeftijd, omgevingsfactoren en pathologische omstandigheden kunnen de snelheid en de mate van de individuele mechanismen beïnvloeden en moeten daarom ook in acht genomen worden bij vergelijk van de werking in verschillende omstandigheden en zeker waar het voorspellingen van de werking van het geneesmiddel in de mens zou betreffen. In **hoofdstuk 2** is een samenvatting gegeven van de ziekte van Parkinson en van de geneesmiddelen die gebruikt worden bij de behandeling van deze ziekte. Verder zijn de mechanismen en factoren en hun mogelijke invloed op de centrale werking van geneesmiddelen beschreven. De nadruk is hierbij op de bijdrage van de BHB onder pathologische omstandigheden gelegd.

3. Diermodellen als gereedschap in onderzoek naar geneesmiddelen voor de ziekte van Parkinson

De ziekte van Parkinson bestaat uit meerdere componenten die elk veroorzaakt worden door een samenspel van genetische en niet-genetische factoren. In de ontwikkeling van nieuwe geneesmiddelen voor de ziekte van Parkinson is het daarom belangrijk om de invloed van het geneesmiddel op het gehele biologische systeem te bestuderen (systeemfarmacologie). Diermodellen zijn een essentieel

onderdeel van systeemfarmacologisch onderzoek, zoals het onderzoek naar de functionaliteit van de BHB onder verschillende omstandigheden (bijv. ziekte). Diermodellen vormen een belangrijk onderdeel van geïntegreerde onderzoeksmethoden die nodig zijn om de individuele componenten volledig te kunnen begrijpen, inclusief hun eventuele tijdsafhankelijkheid. Informatie verkregen uit dit onderzoek kan gebruikt worden voor de ontwikkeling van mechanistische PK-PD modellen. Het doel van het gebruik van mechanistische PK-PD modellen is het verbeteren van de karakterisatie en de voorspelbaarheid van processen die betrokken zijn bij de geneesmiddelconcentratie en -werking onder fysiologische alsook pathologische condities. Een belangrijk onderdeel van mechanistische PK-PD modellering is het expliciete onderscheid tussen parameters die specifiek zijn voor het geneesmiddel en parameters die specifiek zijn voor het biologische systeem (mens, dier). De parameters die specifiek zijn voor het geneesmiddel kunnen vaak via *in vitro* experimenten verkregen worden, terwijl de biologische systeemparameters door middel van *in vivo* experimenten, bijvoorbeeld in diermodellen, bepaald moeten worden omdat dit begrijpelijkerwijs veelal niet in de mens kan gebeuren.

Diermodellen zijn daarom essentieel in het onderzoek naar de (veranderingen in) mechanismen die een rol spelen in de relatie tussen PK en PD van een geneesmiddel, zo ook voor de functionaliteit van de BHB onder verschillende omstandigheden.

Diermodellen die gebruikt worden in onderzoek naar de ziekte van Parkinson, kunnen worden onderverdeeld in toxine-geïnduceerde modellen en genetische modellen. In **hoofdstuk 3** zijn deze diermodellen met hun belangrijkste eigenschappen samengevat. Hierbij zijn de toxine-geïnduceerde modellen verder uitgelicht.

De meest toegepaste toxine-geïnduceerde diermodellen voor de ziekte van Parkinson, maken gebruik van 6-hydroxydopamine (6-OHDA), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), paraquat, maneb of rotenon. Verschillende soorten diermodellen zijn nodig om verschillende onderzoeksvragen te kunnen beantwoorden. De meeste diermodellen beschreven in **hoofdstuk 3**, hebben betrekking op een acute inductie van de ziekte van Parkinson waarbij de dopamine-producerende zenuwcellen in korte tijd en in grote mate afsterven. Het neurotoxine rotenon kan als één van de weinigen de ziekte van Parkinson langzaam induceren, waardoor de voortschrijding van de ziekte beter nagebootst kan worden. Rotenon heeft als bijkomend voordeel boven de andere toxine-geïnduceerde diermodellen dat het de vorming van Lewy

bodies in de substantia nigra tot gevolg kan hebben, vergelijkbaar met wat er gebeurt bij de ziekte van Parkinson in de mens. Het rotenon diermodel lijkt daarom geschikt voor gebruik ten behoeve van het ontwikkelen van mechanistische PK-PD modellen waarin ook de voortschrijding van de ziekte als factor meegenomen kan worden. Het onderzoek zoals verder beschreven in dit proefschrift heeft zich daarom gericht op het evalueren van het diermodel voor de ziekte Parkinson met rotenon.

Als maat voor het effect van een nieuw geneesmiddel wordt vaak gebruik gemaakt van de verandering in het gedrag van het diermodel. Voor diermodellen van de ziekte van Parkinson zijn verschillende gedragsmetingen mogelijk. De meest voorkomende is het amfetamine-geïnduceerd rotatiegedrag. Andere methoden meten bijvoorbeeld de voortbeweging of de grijpkracht. **Hoofdstuk 3** geeft een samenvatting van de meest gebruikte gedragsmethoden in diermodellen van de ziekte van Parkinson. Voorts is intracerebrale microdialyse beschreven als een zeer geschikte techniek voor het bepalen van BHB functionaliteit. Dit omdat met deze techniek tijdsafhankelijke concentratiemetingen kunnen worden verricht die nodig zijn voor het bepalen van de mate en snelheid van mechanismen van BHB transport van een bepaalde stof (geneesmiddel).

4. Rotenon als neurotoxine om de ziekte van Parkinson te induceren in de rat

In de eerste studie beschreven in **hoofdstuk 4** werd rotenon via een osmotisch minipompje subcutaan toegediend aan mannelijke Lewis ratten en het effect op gewicht en gedrag bepaald terwijl de permeabiliteit van de BHB voor de marker fluoresceine met behulp van microdialyse werd gemeten. Post-mortem werd de mate van schade aan de zenuwcellen in het striatum en de substantia nigra bepaald door een immunokleuring van de hersenen op tyrosine hydroxylase (TH), als maat voor de dopaminerge cellen. Ook werd de schade aan perifere organen zoals o.a het hart, de lever, nieren en de maag bepaald. De gegevens van de rotenon behandelde ratten werden vergeleken met die zonder behandeling (controle). De resultaten toonden aan dat het subcutaan toegediende rotenon nauwelijks of geen schade toebracht aan de dopamine-producerende zenuwcellen in het striatum of substantia nigra, maar wel in grote mate aan de perifere organen. Tevens bleek er door subcutaan toegediend rotenon verandering in de BHB permeabiliteit en motoriek te onstaan, echter niet ten gevolge van inductie

van de ziekte van Parkinson, maar door aspecifieke toxiciteit. Deze resultaten zijn later door andere onderzoeksgroepen bevestigd.

De subcutane toediening van rotenon bleek dus niet geschikt voor het induceren van de ziekte van Parkinson. Gezocht werd naar een alternatieve manier van toediening. Er kwamen resulaten beschikbaar van studies waarbij rotenon intracerebraal in de substantia nigra of de medial forebrain bundle (MFB) werd geïnjecteerd. Hierbij was een afname te zien in de dopamine concentraties in het striatum en de substantia nigra. Ook gedrag, specifiek voor de ziekte van Parkinson werd in deze experimenten waargenomen, maar gegevens van TH immunokleuring ontbraken. Dit gold als basis voor de tweede set experimenten beschreven in **hoofdstuk 4** waarin rotenon direct en in één hersenhelft plaatselijk in de MFB van de rat werd toegediend, teneinde perifere toxiciteit te vermijden. De andere hersenhelft diende als controle. Er werden drie doses rotenon (0.5, 2.0 and 5.0 µg) getest voor het vinden van een 'optimale' dosis voor het induceren van de ziekte van Parkinson. Het effect van de intracerebrale toediening op gewicht en perifere orgaan toxicologie werd gemeten voor vergelijking met de resultaten verkregen na subcutane toediening van rotenon. Verder werd de BHB permeabiliteit gemeten, alsook het rotatiegedrag van de rat na toediening van amfetamine. Post-mortem bleek er specifieke schade te zijn ontstaan aan de dopaminerge zenuwcellen in het striatum en de substantia nigra, zonder enige aanwijzigingen voor perifere toxicologie. Incidenteel konden in de substantia nigra alfasynucleïne ophopingen aangetoond worden. Deze bleken echter niet direct gerelateerd te zijn aan de mate van schade aan de dopaminerge cellen. Verder nam de schade aan de dopaminerge zenuwcellen toe met de tijd na toediening van rotenon, met name de 5.0 µg dosis, terwijl de afname in TH-kleuring bleek een plateau te bereiken op dag 28 na de injectie van rotenon, als laatste meetpunt. Ook werd een grote toename in het amfetamine geïnduceerd rotatiegedrag gevonden. Er waren echter geen verschillen gezien in BHB permeabiliteit. Er werd geconcludeerd dat met het intracerebraal toedienen van rotenon voorziet in een ratmodel van de ziekte van Parkinson met een progressief verloop van de ziekte.

5. De toepassing van het intracerebrale rotenon rat model: L-DOPA kinetiek

In **hoofdstuk 5** werd in het intracerebrale rattenmodel van de ziekte van Parkinson (zoals beschreven in **hoofdstuk 4**) de kinetiek van L-DOPA in plasma en in de hersenen onderzocht, alsmede de effecten van L-DOPA op de dopamine metabolieten 3,4-dihydroxyphenylacetic acid (DOPAC) en homovanillic acid (HVA) in de extracellulaire vloeistof van de hersenen, op 14 dagen na injectie van rotenon (5 µg). L-DOPA is de precursor van dopamine dat weer gemetaboliseerd wordt naar DOPAC of HVA. DOPAC kan buiten de cel worden geconjugeerd naar een glucuronide of omgezet worden naar HVA. Ongeconjugeerd HVA is daarmee het belangrijkste eindprodukt van dopamine in de hersenen van primaten. In de hersenen van ratten is dit DOPAC. In het experiment beschreven in hoofdstuk 5 werd L-DOPA in 3 verschillende doses intraveneus toegediend (10, 25 of 50 mg/kg). Er werden serieel bloedmonsters afgenomen om de PK van L-DOPA in plasma te bepalen. Met behulp van intracerebrale microdialyse werden L-DOPA, dopamine, DOPAC en HVA in de rotenon-geinjecteerde hersenhelft en de controle hersenhelft bepaald en vergeleken. Ratten werden op basis van post mortem TH immunokleuring, ten opzichte van controle hersenhelft, onderverdeeld in "non-responders" (geen verandering) "responders" (verlaging, blijkens altijd tot minder dan 40%). Deze gegevens werden allen gebruikt voor het ontwikkelen van een populatie-PK model met behulp van NONMEM software. Dit ontwikkelde populatiemodel is de eerste waarbij zowel de PK van L-DOPA in plasma en in de hersenen in zowel gezonde en zieke condities, alsook de effecten van L-DOPA op de concentraties van DOPAC en HVA, worden beschreven. Samengevat geeft het model aan dat de PK van L-DOPA in plasma en in de hersenen niet wordt beinvloed door de ziekte condities, terwijl duidelijke verschillen in de eliminatiesnelheidsconstantes van DOPAC en HVA werden gevonden. Dit kan op basis van kinetische principes worden teruggevoerd op verlaagde concentraties van dopamine in de zieke hersenhelft.

6. Discussie en toekomst perspectieven

Het doel van de studies beschreven in dit proefschrift was te onderzoeken of het gebruik van het neurotoxine rotenon in ratten geschikt was voor het induceren van een meer chronisch en progessief verloop van de ziekte van Parkinson ten behoeve van onderzoek naar de relatie tussen farmacokinetiek (PK) en farmacodynamiek (PD) van geneesmiddelen die worden gebruikt voor de

behandeling van de ziekte van Parkinson. Hierbij werd speciaal de nadruk gelegd op mogelijke wijzigingen in snelheid en mate van een aantal transport-mechanismen van de bloed-hersenbarrière (BHB) ten gevolge van de pathologische condities.
De resultaten toonden aan dat het subcutaan toegediende rotenon nauwelijks of geen schade toebracht aan de dopamine-producerende zenuwcellen, maar wel in grote mate aan de perifere organen. Tevens bleek er door subcutaan toegediend rotenon verandering in de BHB permeabiliteit en motoriek te onstaan, echter niet ten gevolge van inductie van de ziekte van Parkinson, maar door aspecifieke toxiciteit. Deze resultaten zijn later door andere onderzoeksgroepen bevestigd.

Intracerebraal toegediend rotenon (met name de 5.0 µg-dosis) levert een progressief rat model voor de ziekte van Parkinson, met een plateau in de afname in TH kleuring op dag 28 na toediening en met incidenteel alfasynucleïne ophopingen. Deze alfasynucleïne ophopingen, een kenmerk van de ziekte van Parkinson bij de mens, waren nog niet eerder aangetoond voor studies met intercerebraal toegediend rotenon. Verder onderzoek is nodig om te bepalen of er na een enkele intracerebrale toediening van rotenon, na 28 dagen sprake is van een verdere progressie van de ziekte, en of er met de tijd meer ophopingen van alfasynucleïne aanwezig zullen zijn. Daarin zal ook het leeftijdsverschil als zodanig meegenomen moeten worden. Veel studies hebben namelijk aangetoond dat er leeftijds-gerelateerde veranderingen zijn in neurochemie en neurobiologie. Zo zijn er humane- en diergegevens over de veranderingen in de dopaminerge neurotransmitter pathway. Verder blijkt dat met toenemen van de leeftijd ook de gevoeligheid van dopamine-producerende zenuwcellen voor rotenon toeneemt zoals werd aangetoond in de rat.

De experimenten in dit proefschrift voor het bepalen van BHB transportmechanismen zijn uitgevoerd op 14 dagen na toediening van rotenon en lieten noch ziekte-geïnduceerde veranderingen in BHB permeabiliteit (passief) noch verandering in het BHB influx transport van L-DOPA door de LAT1 transporter (actief) zien. Wel werden duidelijke verschillen waargenomen in de kinetiek van DOPAC en HVA in het striatum van de zieke hersenhelft ten opzichte van de gezonde. Het is niet uitgesloten dat er op andere locaties in het brein mogelijk wel veranderingen in BHB functionaliteit ten gevolge van het ziekteproces optreden. Andere onderzoeksgroepen hebben verhoogde BHB permeabiliteit aangetoond in het 6-OHDA model voor de ziekte van Parkinson,

die focaal van aard was. Het is dan ook zinvol om BHB transport op andere momenten op de tijdschaal van ziekteprogressie het intracerebrale rotenon model verder te onderzoeken.

Het aantal aanwijzingen in de literatuur dat de functionaliteit van de BHB is veranderd in de ziekte van Parkinson groeit nog steeds en geeft aan dat onderzoek naar BHB karateristieken in deze ziekte erg zinvol is. Bromocriptine is een D2 agonist en geneesmiddel die wordt toegepast bij de behandeling van de ziekte van Parkinson. Het is ook een substraat voor P-gp en gegeven het feit dat PET studies hebben aangetoond dat de werking van P-gp in patiënten met Parkinson verminderd zou zijn, is het erg zinvol om de PK en PD van bromocriptine in het intracerebrale rotenon rat model te bestuderen. Een tweede D2 agonist die gebruikt wordt bij de behandeling van de ziekte van Parkinson is pramipexole. Dit is een geneesmiddel dat zowel via passieve difussie als door een actieve transporter de BHB passeert. Dit geneesmiddel zou ook een interessante stof zijn om ziekte-geïnduceerde veranderingen in BHB transport bij de ziekte van Parkinson te onderzoeken.

7. Conclusie

Het intracerebraal toedienen van rotenon in de MFB voorziet in een ratmodel van de ziekte van Parkinson met een progressief verloop. In dit model werd, 14 dagen na toediening van rotenon, geen veranderingen in BHB permeabiliteit gevonden, en ook geen veranderingen in het actieve BHB transport van L-DOPA door de LAT1 transporter. Wel werden duidelijke verschillen waargenomen in de kinetiek van DOPAC en HVA in het striatum van de zieke hersenhelft ten opzichte van de gezonde, wat duidt op een vermindering in de dopamine concentratie in de zieke hersenhelft zoals kenmerkend is voor de ziekte van Parkinson. Er kan worden geconcludeerd dat het intracerebrale rotenon ratmodel een goed bruikbaar diermodel is in het systeemfarmacologisch onderzoek naar de factoren die de effectiviteit van anti-parkinson middelen in verschillende stadia van de ziekte bepalen.

Nawoord

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Curriculum Vitae

Paulien Ravenstijn werd geboren op 4 juli 1977 te Terneuzen. Na het behalen van het Europese Baccalaureaat aan de Europese School te Karlsruhe in Duitsland, begon zij in 1996 aan de studie Bio-Farmaceutische Wetenschappen aan de Universiteit Leiden, waar zij in 1998 het propedeutisch diploma verkreeg. Tijdens de doctoral fase heeft zij haar hoofdvakstage gedaan op de afdeling 'Drug Delivery Technology' van het Leiden/Amsterdam Center for Drug Research, onder begeleiding van dr. B.I. Florea en dr. G. Borchard. Het verslag van haar stage was getiteld 'Trimethylchitosan-oligomer as a Transfection Vector for Pulmonary Gene Therapy: Biophysical Characteristics and Transfection Ability'. In oktober 2001 behaalde zij haar doctoraal diploma Bio-Farmaceutische Wetenschappen.
Van oktober 2001 tot en met september 2006 was zij als assistent in opleiding verbonden aan de afdeling Farmacologie van het Leiden/Amsterdam Center for Drug Research onder begeleiding van dr. E.C.M. de Lange en Prof. dr. M. Danhof, alwaar het in dit proefschrift beschreven onderzoek werd uitgevoerd. Sinds oktober 2006 is de auteur werkzaam als scientist Clinical Pharmacokinetics bij Johnson & Johnson Pharmaceutical Research and Development in België.

Paulien Ravenstijn was born on July 4, 1977 in Terneuzen, The Netherlands. In 1996 she received the European Baccalaureate at the European School in Karlsruhe, Germany and commenced with the study Bio-Pharmaceutical Sciences at Leiden University, The Netherlands. During her studies, she performed a scientific internship entitled 'Trimethylchitosan-oligomer as a Transfection Vector for Pulmonary Gene Therapy: Biophysical Characteristics and Transfection Ability' at the division of Drug Delivery Technology of the Leiden/Amsterdam Center for Drug Research under supervision of dr. B.I. Florea en dr. G. Borchard. In 2001 she obtained her Master of Science degree and started as a PhD student at the division of Pharmacology of the Leiden/Amsterdam Center for Drug Research, under supervision of dr E.C.M. de Lange and Prof. dr. M. Danhof, which led to this PhD thesis.
Currently, Paulien Ravenstijn is employed as scientist Clinical Pharmacokinetics at Johnson & Johnson Pharmaceutical Research and Development in Belgium.

List of Publications

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